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DETERMINANTS OF RECURRENT MELIOIDOSIS



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Thesis submitted in the total fulfillment of the degree of

Doctor of Philosophy

The Open University

Supervisors: Dr Sharon Peacock,
Dr Kasia Stepniewska and Prof Nicholas Day

Sponsoring establishment: Mahidol-Oxford Tropical
Medicine Research Unit, Thailand

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Glossary of terms.

AHL	<i>N</i> -acyl-homoserine lactone
AMC	amoxicillin-clavulanic acid
<i>bsa</i>	<i>Burkholderia</i> secretion apparatus
CDC	Centers for Disease Control
CFU	colony forming units
CHEF	contour-clamped homogenous electrophoresis field
CI	confidence interval
CRP	C-reactive protein
CSF	cerebrospinal fluid
CT	computed tomography
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EPS	exopolysaccharide
<i>et al.</i>	and others
G-CSF	granulocyte-colony stimulating factor
GFR	glomerular filtration rate
HR	hazard ratio
ICU	intensive care unit
IFA	indirect immunofluorescence assay
IHA	indirect haemagglutination assay
IQR	interquartile range
ISRCTN	International Standard Randomised Controlled Trial Number
LD50	lethal dose, 50%

LPS	lipopolysaccharide
MLST	multilocus sequence typing
MRI	magnetic resonance imaging
N/A	not available
OR	odds ratio
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
PHB	polyhydroxybutyrate
<i>pilA</i>	putative pilus structural protein
RAPD	random amplified polymorphic DNA
RCT	randomised controlled trial
RFLPs	restriction fragment length polymorphisms
RR	relative risk
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulfate
SPS	sodium polyanethol sulfonate
ST	sequence type
TSB	trypticase soy broth
TTSS	type III secretion system

Publication arising from thesis.

- (1) Maharjan B, Chantratita N, Vesaratchavest M, Cheng A, Wuthiekanun V, Chierakul W, Chaowagul W, Day NP, Peacock SJ. Recurrent melioidosis in patients in northeast Thailand is frequently due to reinfection rather than relapse. *J Clin Microbiol* 2005; 43(12):6032-6034.
- (2) Limmathurotsakul D, Wuthiekanun V, Chantratita N, Wongsuvan G, Thanwisai A, Biaklang M, Tumapa S, Lee S, Day NP, Peacock SJ. Simultaneous infection with more than one strain of *Burkholderia pseudomallei* is uncommon in human melioidosis. *J Clin Microbiol* 2007; 45(11):3830-3832.
- (3) Chantratita N, Wuthiekanun V, Limmathurotsakul D, Vesaratchavest M, Thanwisai A, Amornchai P, Tumapa S, Feil EJ, Day NP, Peacock SJ. Genetic Diversity and Microevolution of *Burkholderia pseudomallei* in the Environment. *PLoS Negl Trop Dis* 2008; 2(2):e182.
- (4) Limmathurotsakul D, Chaowagul W, Chierakul W, Stepniewska K, Maharjan B, Wuthiekanun V, White NJ, Day NP, Peacock SJ. Risk factors for recurrent melioidosis in northeast Thailand. *Clin Infect Dis* 2006; 43(8):979-986.
- (5) Limmathurotsakul D, Chaowagul W, Chantratita N, Wuthiekanun V, Biaklang M, Tumapa S, White NJ, Day NP, Peacock SJ. A simple scoring system to differentiate between relapse and re-infection in patients with recurrent melioidosis. (manuscript in preparation).

Abstract.

Recurrent melioidosis represents relapse following failure to eradicate bacteria responsible for the primary infection or re-infection with a new strain. The first results chapter (chapter 3) evaluates the proportion of recurrent melioidosis due to relapse versus re-infection. Isolates from the same patient with an identical genotype were considered as relapse and those with a different genotype as re-infection. Three quarters of recurrent cases were due to relapse and one quarter were due to re-infection. There are two ways in which this approach could be confounded. First, 're-infection' could actually represent relapse if primary infection was caused by simultaneous infection with multiple *B. pseudomallei* strains, followed by chance selection of different strains from the two episodes for genotyping. The chance of this mistake occurring is based on the rate of polyclonal *B. pseudomallei* infection. Chapter 4 describes the rate of polyclonal infection in a large group of unselected patients in northeast Thailand, which was very low (2/133 cases, 1.5%). Second, 'relapse' could actually represent re-infection in the event that re-infection was caused by a *B. pseudomallei* strain that was by chance identical to the primary strain. The probability of this happening is based on the degree of genetic diversity of *B. pseudomallei* in the environment. Chapter 5 demonstrates that the population of *B. pseudomallei* in even a small sampling site is extremely diverse. Thus, it is unlikely that the assessment of the causes of recurrent melioidosis contained significant errors due to polyclonal infection or low genetic diversity of the organism. Chapter 6 examines specific risk factors of relapse and re-infection. Duration and choices of antibiotics used for the primary episode were major determinants of relapse. Chapter 7 compares the clinical manifestations of relapse and re-infection and develops a simple scoring index to predict relapse or re-infection in patients presenting with recurrent melioidosis.

Chapter 1. Introduction.

1.1 General background.

1.1.1 The genus *Burkholderia*.

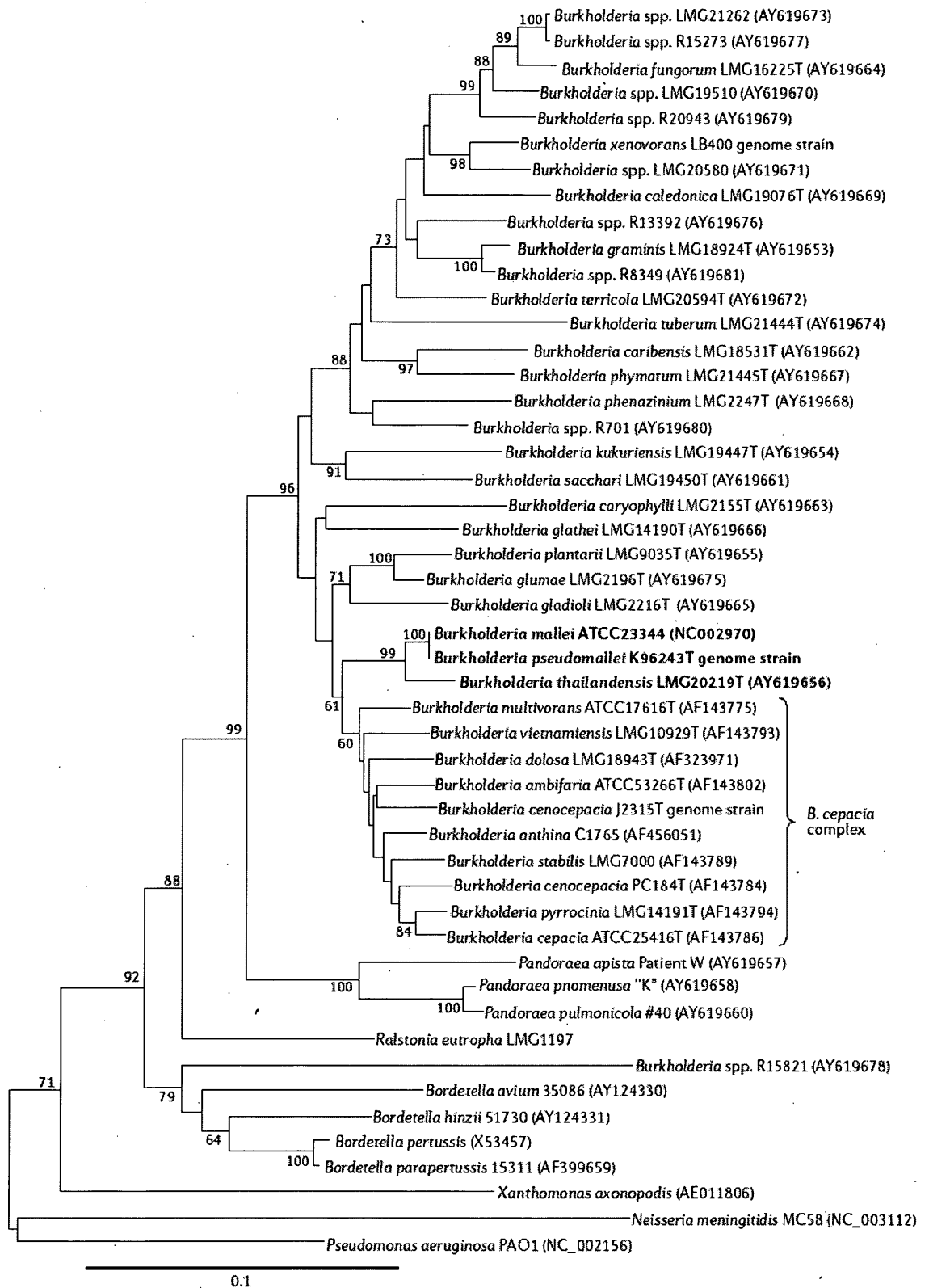
Burkholderia spp. are glucose non-fermenting Gram-negative bacilli that were formerly classified within the genus *Bacillus*, *Malleomyces* and *Pseudomonas*, only to be regrouped as a new genus in 1992 (271). The genus *Burkholderia* contains over 30 species, which occupy remarkably diverse ecological niches ranging from soil to the human respiratory tract (58). *Burkholderia thailandensis* and *Burkholderia pseudomallei* are saprophytes, and both may be isolated from the same environment.(236). The *Burkholderia cepacia* complex is ubiquitous in nature and can be found in soil, water (including sea water), the rhizosphere of plants, in humans and various animal species and even in the hospital environment.

Some *Burkholderia* species are plant pathogens (58). *Burkholderia caryophylli* is pathogenic for carnations (*Dianthus caryophyllus*) and causes onion rot. *Burkholderia plantarii* causes seedling blight of rice and forms a disease-causing aromatic compound. *Burkholderia glumae* causes rot of rice grains and seedlings. *Burkholderia andropogonis* is the causative agent of stripe disease of sorghum (*Andropogon* sp.), and leaf spot of velvet bean (*Stizolobium deeringianum*). By contrast, several *Burkholderia* species have beneficial interactions with plants and colonize roots, stems and leaves. *Burkholderia vietnamiensis* and *Burkholderia kururensis* act as N₂-fixing bacteria for maize and coffee plants (87). *Burkholderia tuberum* and *Burkholderia phymatum* are involved in nitrogen fixation and nodulation in tropical legume plants.

There are three important human pathogenic species. These are *Burkholderia mallei* which causes glanders, *Burkholderia pseudomallei* which causes melioidosis,

and *Burkholderia cepacia* which is a cause of pulmonary infection in patients with cystic fibrosis. A phylogenetic tree based on *recA* sequences of the genus *Burkholderia* is presented in figure 1-1 (170).

Figure 1-1. The phylogeny of the *Burkholderia* genus.



1.1.1.1 *Burkholderia pseudomallei*.

B. pseudomallei is a Gram-negative, aerobic, non-spore-forming, facultatively intracellular, oxidase-positive motile bacillus. This bacterium is the causative agent of melioidosis and is classified as a Hazard Group 3 pathogen by the Centers for Disease Control (CDC), United States. In clinical material, Gram staining may be irregular, and bipolar staining may be seen. *B. pseudomallei* accumulates polyhydroxybutyrate (PHB) in large, central granules to give a negative staining effect on Gram stain. However, this appearance is not specific to *Burkholderia* and may be absent in young colonies. The accumulation of prominent granules of PHB as an energy store reflects a metabolism adapted to long-term survival (109). The bacilli usually appear singly but may be in long bundles. During environmental sampling, *B. pseudomallei* can be distinguished from the closely related but less pathogenic *B. thailandensis* by the ability of the latter to assimilate L-arabinose (205). The genome sequence of *B. pseudomallei* strain K96243 from Thailand was completed in 2004. This demonstrated that *B. pseudomallei* contains many genes that promote survival in diverse and challenging environments and a large selection of genes that modulate pathogenicity and host-cell interactions (109).

1.1.1.2 *Burkholderia mallei*.

Burkholderia mallei is a non-motile, usually oxidase-negative, Gram-negative bacillus that has been classified as a Hazard Group 3 pathogen. The bacilli may be arranged singly, in pairs end to end, in parallel bundles or palisades. *B. mallei* is a host-adapted pathogen that does not persist in nature outside of its host. It is an obligate parasite of horses, mules, and donkeys with no other known natural reservoir. This organism is the causative agent of glanders (212), which is one of the oldest diseases known to man and was first described by Aristotle (384-322 before Christ) (165).

Glanders is an abscess-forming infection of horses and other equines, which occasionally affects humans with occupations involving close contact with infected animals. In humans, glanders is characterized by pustular skin lesions, signs of sepsis, pneumonia and either necrosis of the tracheobronchial tree if the organism was inhaled, or multiple abscesses if the skin was the portal of entry. Glanders was eradicated from North America in the 1930's and is very rare today. There is evidence that *B. mallei* was also used as a biological weapon (254). The complete genome sequence of *B. mallei* strain ATCC23344 was published in 2004. This identified that major genetic adaptation had occurred in association with a putative shift from soil saprophyte to a highly specialized obligate mammalian pathogen (165). The major genetic feature was genomic downsizing. Whole genome sequence confirmed a previous suggestion based on multilocus sequence typing (MLST) that *B. mallei* has evolved from a clone of *B. pseudomallei* (98).

1.1.1.3 *Burkholderia cepacia*.

Burkholderia cepacia was discovered from rotting onions by Walter Burkholder in 1949. However, it is now considered by agricultural microbiologists as an agent that promotes crop growth (59). *B. cepacia* is motile by virtue of polar flagella. It is found in soil, water and on plants, and can survive longer in wet environments than in dry ones. *B. cepacia* has been used as a biocontrol agent as it is not toxic, does not pollute water or soil, can degrade unwanted chemicals and produces multiple antibiotics against fungi that are pathogenic for plants (111). After widespread usage, this organism was found to be a human pathogen. It was first detected in cystic fibrosis patients in 1971 (112). *B. cepacia* is now recognized as an important opportunistic pathogen in the hospital setting and an important pathogen in cystic fibrosis patients (101). Cystic fibrosis patients who acquire *B. cepacia* may follow one of three different

clinical courses: persistent colonization without alteration of lung function, significantly accelerated reduction of lung function, or rapid deterioration with acute necrotic pneumonia and fatal outcome within several weeks or months, which has been termed cepacia syndrome (124).

1.1.1.4 *Burkholderia thailandensis*.

Burkholderia thailandensis was first recognized in 1997 as a non-virulent biotype of *Burkholderia pseudomallei* that was distinguishable from a virulent biotype by the ability to assimilate arabinose (205). *B. thailandensis* is Gram-negative, motile, and has two to four flagella. *B. thailandensis* has similar morphology and antigenicity to *B. pseudomallei*. The API20NE and API50CH biochemical profiles used to differentiate glucose fermenting Gram negative bacteria give similar profiles for *B. thailandensis* and *B. pseudomallei* (267). These two species also react similarly in the indirect haemagglutination assays (IHA) used for melioidosis serology (267). Lipopolysaccharide (LPS) from *B. thailandensis* and *B. pseudomallei* is also immunologically indistinguishable (8). However, the virulence of these two organisms is totally different; the 50% lethal dose (LD₅₀) for *B. thailandensis* was reported to be 10⁹ CFU/mouse, compared with 182 CFU/mouse for *B. pseudomallei* (205). *B. thailandensis* may also be found in large quantities in the environment in the same soil or water as *B. pseudomallei* (236). Approximately 25% of soil isolates from northeast Thailand are culture positive for *B. thailandensis* (267). Colonial morphologies of both species grown on the same selective agar may be difficult to distinguish. A latex agglutination test based on a monoclonal antibody to *B. pseudomallei* exopolysaccharide (EPS) can be used to rapidly differentiate *B. pseudomallei* and *B. thailandensis* (positive for *B. pseudomallei* but negative for *B. thailandensis*) (262). Only 2 cases of infection by *B. thailandensis* have been reported. The strain from the

first case report was lost and cannot be re-evaluated (144). The other case reported in the US was 2-year old boy with pneumonia and bacteraemia caused by *B. thailandensis*. This patient was pulseless and apneic following a road traffic accident in which he was immersed in water for 2 minutes (95). The complete genome sequence of *B. thailandensis* strain E264 was published in 2005. This suggested that divergent evolution of a small set of genes was associated with the development of different life styles among *B. thailandensis*, *B. pseudomallei* and *B. mallei* (134).

1.1.2 Bacteriology of *B. pseudomallei*.

1.1.2.1 *B. pseudomallei* as an environmental saprophyte.

B. pseudomallei is commonly found in water and soil in melioidosis-endemic areas including northern Australia and Southeast Asia, and is occasionally found in other tropical areas of the world (33). *B. pseudomallei* has the unusual ability to survive for months to years in the environment throughout the dry season (233) and in water in the absence of nutrients (269). Bacterial counts of *B. pseudomallei* in soil have been shown to be related to the risk of developing melioidosis (206). *B. pseudomallei* contaminating a community water supply has been reported to have caused two outbreaks of melioidosis (70;122).

Periodic soil sampling for *B. pseudomallei* has shown that the persistence of culturable bacteria depends on soil type and hydration (233). Well drained, light, sandy soils are less able to support the persistence of *B. pseudomallei*, while waterlogged, heavy clay soils are much better at supporting bacterial persistence (123). The optimal temperature and pH for *B. pseudomallei* is 24°C to 32°C and 5 to 8, respectively (235). Soil with a water content of less than 10% led to the death of *B. pseudomallei* within 70 days, while soil with a water content of more than 40% supported the persistence of *B. pseudomallei* for more than 2 years (235). Soil samples taken from a depth greater than

30 centimeters were more likely to contain *B. pseudomallei* compared with the surface, reflecting a preference for higher moisture content and lower temperature (233;268). Samples recovered from a soil depth of 90 cm in Thailand yielded up to 10^5 CFU *B. pseudomallei* per gram of soil (206). A rising water table in the tropical rainy season has been proposed as one explanation for the reappearance of *B. pseudomallei* in the superficial layers of soil. There is a strong association between monsoonal rains, occupational exposure to surface water and mud, and the incidence of *B. pseudomallei* infection (67;222).

1.1.2.2 *B. pseudomallei* genome.

The published genome of *B. pseudomallei* strain K96243 contains two chromosomes of 4.07 megabase pairs and 3.17 megabase pairs, respectively (109). The large chromosome encodes many core functions associated with central metabolism and bacterial growth. The small chromosome carries more genes associated with adaptation and survival in different niches. The G+C content is 68%. A summary of the key genes identified is listed in table 1-1.

Table 1-1. Genes associated with survival and virulence functions encoded by the *B. pseudomallei* genome.

Survival	
Secondary metabolism	Possible antibiotic, surfactant and siderophore biosynthesis pathways
Drug resistance	Ambler class A, B, and D β -lactamases, including cephalosporinase and oxacillinase; multidrug efflux systems, including aminoglycoside-, macrolide-, and polymyxin β -specific systems; aminoglycoside acetyltransferase
Intracellular stress	Superoxide and nitric oxide detoxification
Motility and chemotaxis	Flagellar system, chemotaxis-associated proteins
Virulence	
Secretion	Type I, type II, type III, and type V protein secretion systems, including three type III secretion systems
LPS and capsule	Capsular polysaccharide synthesis and export cluster; LPS biosynthesis cluster.
Exoproteins	Phospholipases C, metalloprotease A, and other proteases and collagenases
Adhesins	Surface proteins potentially modulate host-cell interactions
Fimbriae and pili	Type I fimbriae, type IV pili and <i>tad</i> -type pili

Genomic islands are likely to represent DNA that has been recently horizontally acquired and are variably found in clinical and environmental strains of *B.*

pseudomallei. Approximately 6% of the *B. pseudomallei* genome is made up of putative genomic islands (109). These are mostly absent from the *B. thailandensis* and *B. mallei* genomes. Gene deletion may have accounted for their absence in *B. mallei*, possibly as a result of mammalian host restriction and the associated change in selective pressure (165).

1.1.2.3 Virulence factors of *B. pseudomallei*.

B. pseudomallei is a facultative intracellular pathogen which can invade and survive in eukaryotic cells, even in phagocytes (180). After internalization, *B. pseudomallei* can escape from a membrane bound phagosome into the cytoplasm (127). This is followed by actin polymerization and the formation of membrane protrusions at one pole, a process that mediates cell-to-cell spread of the organism (216). *B. pseudomallei* also has the ability to induce cell fusion and multinucleated giant cell formation, and the ability to induce apoptosis in both phagocytic and nonphagocytic cells (132). These characteristics may be important in human infection and virulence. Additional putative virulence factors for infection are quorum sensing, capsular polysaccharide and pili, LPS and the ability to form biofilm.

1.1.2.3.1 Quorum sensing.

This term describes the ability by bacteria to communicate and display coordinated behaviour through the effect of a secreted signaling molecule. Quorum sensing networks, often of a highly complex nature, have been identified in numerous Gram-negative bacterial pathogens. *B. pseudomallei* has been reported to co-ordinate gene expression through the expression of an *N*-acyl-homoserine lactone (AHL) (237).

Disruption of the operon encoding its expression led to a significant increase in LD₅₀ in hamsters after intraperitoneal challenge, and an increase in time to death and decrease in organ colonization after aerosolized challenge (237). These findings suggest that quorum sensing plays an important role in virulence.

1.1.2.3.2 Type III secretion system.

B. pseudomallei contains three type III secretion system (TTSS) gene clusters (109). One of these clusters (TTSS3 cluster) shares homology with the Inv/Mxi/Spa-like type III secretion gene cluster described for *Salmonella enterica* serovar Typhimurium and *Shigella flexneri*. The related type III secretion gene cluster in *B. pseudomallei* is termed *bsa* (*Burkholderia* secretion apparatus) (216). *bsa* encodes proteins that are very similar to *S. typhimurium* and *S. flexneri* secreted proteins required for invasion, escape from endocytic vacuoles, intercellular spread and pathogenesis. After bacteria escape from endocytic vacuoles into the cytoplasm of infected cells, membrane protrusions are formed through the action of actin polymerization at one pole. This facilitates cell-to-cell spread of bacteria without exposure to antimicrobials or immunoactive molecules. Mutant strains lacking components of the Bsa secretion and translocation apparatus exhibited reduced replication in murine macrophage-like cells, an inability to escape from vacuoles and a complete absence of formation of membrane protrusion and actin tails (216).

1.1.2.3.3 Capsular polysaccharide.

B. pseudomallei produces an extracellular capsular polysaccharide with the structure -3)-2-*O*-acetyl-6-deoxy- β -D-manno-heptopyranose-(1- (186;215). Previously characterized as type I O-polysaccharide of *B. pseudomallei*, this has more recently been considered to be a capsular polysaccharide based on its high molecular mass and

genetic homology with group 3 capsular polysaccharides of other organisms. Capsular polysaccharide is required for *B. pseudomallei* virulence in experimental animal models (17;186). Capsule expression is induced in the presence of serum, and the addition of purified *B. pseudomallei* capsule to serum bactericidal assays increased the survival of *B. pseudomallei* SLR5, a serum sensitive strain, by 1,000-fold (187). Phagocytosis was greater for a capsule-deficient mutant compared to wild-type in the presence of normal human serum (187). Both observations can be explained by the finding that deposition of complement factor C3b on the bacterial cell surface is lower in the presence of capsule (187). The capsule may act as a barrier, blocking access of the complement receptor-1 (CR1) on phagocytes to the C3b deposited on the bacterial surface (187).

1.1.2.3.4 Lipopolysaccharide.

B. pseudomallei lipopolysaccharide (LPS, formerly termed type II O-antigenic polysaccharide) appears to differ in several respects from the LPS of other Gram-negative organisms. *B. pseudomallei* LPS exhibits weaker pyrogenic activity in rodents compared with enterobacterial LPS, but stronger mitogenic activity in murine splenocytes (156). LPS-mediated activation of a mouse macrophage cell line *in vitro* is slower for LPS from *B. pseudomallei* compared with LPS from *E. coli* (238). *B. pseudomallei* LPS appears to be largely conserved across this species. LPS profiling of >700 *B. pseudomallei* isolates using proteinase K digestion and SDS-PAGE silver-stained gels, a technique that examines the O-side chain, demonstrated that the majority had a 'typical' ladder pattern of extracted LPS, 3% had an 'atypical' pattern, and 0.1% do not exhibit a ladder appearance at all (7). The different LPS preparations had similar endotoxic activity in the *Limulus* amoebocyte lysate assay. However, there appears to be a difference in the host immune response to these molecules as there is a lack of immunological cross reactivity on Western blot between typical and atypical LPS using

patient sera infected with typical and atypical LPS isolates (7). LPS of *B. pseudomallei* and *B. thailandensis* have been compared. LPS profiling using proteinase K digestion and SDS-PAGE silver stained gel demonstrated identical ladder patterns for the majority of isolates of both species. The two species exhibited similar immunoblot profiles against pooled sera from patients with melioidosis, and with hyperimmune mouse sera (8). LPS shedding profiles are also similar between the two species (10). This has led to the suggestion that LPS is unlikely to be involved in the virulence and pathogenicity of *B. pseudomallei* (8). Other possible explanations are that: (i) LPS from the two species are antigenically very similar but differ in biological activities, or that (ii) LPS from both species have biological activity *in vivo*, but only *B. pseudomallei* has an additional complement of genes that promotes successful invasion and bacterial dissemination within the host.

1.1.2.3.5 Biofilm.

B. pseudomallei can form a film at the air-liquid interface of a laboratory culture after incubation at 37°C for 24 hours. Biofilms contain slowly growing bacteria and extracellular polysaccharide in complex three-dimensional structures (207). Slow-growing bacteria within the film are inherently more resistant to the action of disinfectants and antibiotics (246). The quantity of biofilm produced by *B. pseudomallei* has been reported to be higher than that of *B. thailandensis* (229). No difference was seen in the LD50 values in BALB/c mice between biofilm-deficient mutant and wild type, indicating that this is not a virulence determinant in this model (229), but this does not rule out a role for biofilm during human disease. Indeed, the development of biofilm *in vivo* could go some way to explaining the reason why even prolonged antibiotic treatment of human melioidosis may fail to eradicate *B. pseudomallei* and resolve infection.

1.1.2.3.6 Flagella.

B. pseudomallei is flagellated and motile. The ability of *B. pseudomallei* to invade and replicate in human lung cells *in vitro* was not different between wild-type and an isogenic mutant defective in flagella expression (53). In one study, there was no difference between an isogenic mutant and wild-type *B. pseudomallei* in diabetic rat and Syrian hamster infection models (79). In a second study, bacterial numbers were markedly reduced in the lung and spleen of BALB/c mice following intranasal infection with an aflagellate mutant compared with wild-type, and the mutant was less virulent following intraperitoneal infection of BALB/c mice as based on LD₅₀ (53).

1.1.2.3.7 Pili.

Adherence is an important virulence factor for bacteria and is mediated by carbohydrate molecules, pilus and non-pilus adhesins. The *B. pseudomallei* genome contains type IV pilin-associated loci, including a gene encoding a putative pilus structural protein (*pilA*). A *pilA* deletion mutant was shown to have reduced adherence to human epithelial cells and was less virulent in a nematode model and a murine model of melioidosis (86). This indicates a possible role for type IV pili in *B. pseudomallei* pathogenesis.

1.1.3 Bacterial genotyping.

A number of molecular typing methods have been used to investigate the epidemiology of *B. pseudomallei*, including ribotyping, random amplified polymorphic DNA (RAPD) analysis, pulsed field gel electrophoresis (PFGE) and MLST.

1.1.3.1 Ribotyping.

Ribotyping is a fingerprinting technique targeting genomic DNA fragments containing all or part of the genes coding for 16S and 23S rRNA. This was originally developed by Stull *et al.* using an *Escherichia coli* rRNA probe to determine the molecular epidemiology of a range of Gram-negative organisms (218). In brief, chromosomal DNA is digested by restriction enzymes such as *EcoRI*, *HindIII*, *Sall*, *PstI* or *BamHI*. The resulting DNA fragments are separated by agarose gel electrophoresis, subjected to southern blot and hybridized with an rRNA gene probe. Hybridized fragments are visualized and the resulting banding pattern defined as a specified ribotype. Ribotype analysis for *B. pseudomallei* was developed in 1993 by Sexton *et al.* (198), in which DNA was digested using *EcoRI* and the rRNA probe was based on *E. coli*. Ribotyping is a poorly discriminative technique for *B. pseudomallei*. For example, in one study conducted in the melioidosis-endemic northern territory of Australia, the majority of isolates belonged to only three different *BamHI* ribotypes (106). This compares with multiple genotypes based on either PFGE or MLST (34;36).

1.1.3.2 Random amplification of polymorphic DNA (RAPD).

RAPD analysis is a PCR-based method using a single short random primer that can anneal to multiple sites and which gives rise to multiple amplification products. PCR products are visualized using agarose gel electrophoresis. Differences in patterns are referred to as restriction fragment length polymorphisms (RFLPs). RAPD was developed for *B. pseudomallei* in 1995 (106). RAPD is less discriminatory than PFGE for several bacterial species (20;136), and suffers from a lack of reproducibility between laboratories.

1.1.3.3 Pulsed Field Gel Electrophoresis (PFGE).

PFGE is a genotyping method used to separate a small number of DNA restriction digest fragments to create a banding pattern of around 15-20 bands. Using standard gel electrophoresis, large DNA fragments (above 50 kb) migrate at similar rates, regardless of size. By repeatedly switching the direction of the electric field, different sized fragments can be separated from each other. In this way, PFGE allows separation of larger pieces of DNA than conventional agarose gel electrophoresis. PFGE is one of the most commonly used typing tools for the study of bacterial epidemiology, and is particularly suited to short-term studies such as the outbreak setting.

Interpreting DNA fragment patterns generated by PFGE is important and guidelines proposed by Tenover *et al.* are often used (230). Isolates that give identical results are classified as “indistinguishable”, rather than “identical”. A banding pattern difference of up to three fragments can arise due to a single genetic event and thus these isolates are classified as “closely related”. Strains with differences of four to six restriction fragments are termed “possibly related”, and strains with more than seven restriction fragments different are considered “different”.

1.1.3.4 Multilocus sequence typing (MLST).

MLST is a molecular typing method based on the sequence comparison of seven conserved housekeeping genes (98). This has the advantage that the output is unambiguous and easily compared between laboratories. MLST has been described for a number of important bacterial pathogens and has become an established technique for the precise characterization of isolates and for epidemiological studies. MLST has been developed for *B. pseudomallei* using the following genes: *ace*, *gltB*, *gmhD*, *lepA*, *lipA*, *nark*, and *ndh*. The different sequences at each of the seven loci are assigned different

allele numbers and the series of seven integers that correspond to the allele numbers at the seven loci define the allelic profile of a strain. As examples, the allele profile 1-1-1-2-4-2-1 defines sequence type (ST) 1, and the allelic profile 1-1-13-1-1-1-1 defines ST10.

1.2 Melioidosis.

The term ‘melioidosis’ was introduced in 1921 by Stanton and Fletcher (214). The name was derived from the Greek ‘melis’ (meaning distemper of asses, which is usually taken to be glanders) and ‘eidos’ (meaning resemblance). Melioidosis is the most common cause of fatal community-acquired bacteraemia in northeast Thailand and bacteraemic pneumonia in the Northern Territory of Australia (67). In northeast Thailand, melioidosis accounts for 20% of all community-acquired bacteraemias, and causes death in 40% of treated patients (222). It mostly infects adults with an underlying predisposing condition, mainly diabetes mellitus and chronic kidney disease. Clinical manifestations of melioidosis are protean and have led to the term “the great mimicker” (273).

1.2.1 Geographical distribution of melioidosis.

The first case of melioidosis in humans was identified in Burma in 1911 (256). The post-mortem of an opium addict conducted by Captain Whitmore and his colleague Krishnaswami at Rangoon General Hospital demonstrated pathological features consistent with a “glanders-like” infection but the causative organism was noted to be motile, which was not consistent with glanders. The causative organism (*B. pseudomallei*) was described as a new species. A large outbreak of melioidosis was subsequently recognised in an animal facility in Malaya in 1913 (214). By 1917, Krishnaswami had reported over 100 human cases from Rangoon (139). Disease in humans and animals was subsequently reported in other areas of Southeast Asia, the region with most of the world’s cases (71). Sporadic cases were reported in the French military in Indo-China, and later in American military personnel returning from Burma and other regions in Southeast Asia during World War II. More than 150 cases of melioidosis occurred in U.S. soldiers involved in the conflict with Vietnam (116).

Reactivation of disease (defined as recurrence of melioidosis some time after acquisition of the bacterium, which sometimes occurred in people with no recollection of a primary episode) in US military personnel many years after leaving the combat zone in Vietnam led to the introduction of the term the “Vietnamese time bomb” (100).

The first reported case of melioidosis in Thailand was in 1935 (48). With a growing clinical recognition and improvement in microbiology facilities, more than 800 cases were reported by 1986 (128). The average annual incidence in northeast Thailand between 1987 and 1991 has been estimated to be 4.4 per 100,000 person-years (222). This disease is now considered an important occupational hazard in Thailand (234). In southern Thailand, melioidosis is not common and the bacterial concentration in soil specimens has been found to be lower compared with the northeast (206;247); however, melioidosis was identified in a number of tsunami survivors who aspirated and/or had lacerations during the 2004 tsunami (47;55). Melioidosis in travellers who visit Thailand or other melioidosis-endemic regions of Asia is also periodically reported (135;151;199).

The first reported human case of melioidosis in Australia was in 1950 (191). Melioidosis is now recognized as the most common cause of fatal community-acquired bacteraemic pneumonia in the Northern Territory of Australia (61). Occasional cases have been reported from Queensland, Western Australia and Papua New Guinea (67). The highest incidence of disease is in the north of the Northern Territory and in the Torres Strait Islands adjacent to Papua New Guinea. A prospective study at the Royal Darwin Hospital during a 10 year period from 1989 to 1999 defined 252 melioidosis patients, giving an estimated average annual incidence of 16.5 per 100,000 (67).

North America and Europe are not melioidosis-endemic areas and cases there represent imported disease. An infection that occurred in an individual in Oklahoma following a farming accident was initially thought to be due to *B. pseudomallei* (270),

but later proved to be caused by a novel species named *Burkholderia oklahomensis* (97). A true case of melioidosis may have occurred in Georgia in association with a motor vehicle accident (167). Outbreaks of melioidosis in animals and two fatal human cases occurred in France in the mid-1970's (71). The source for this outbreak may have been infected horses from Iran or a panda from China. An outbreak of melioidosis in animals was reported in Britain in 1992 following importation of an infected monkeys from the Philippines (74).

Central and South America have recently been recognized as melioidosis-endemic regions. Melioidosis has been reported in Martinique (169), Guadeloupe (173), and Puerto Rico (51;83). The Caribbean is also regarded as endemic for melioidosis (72). Melioidosis has been recognized as an emerging infectious disease in Brazil, where a cluster of cases in Northern Brazil was associated with the onset of unusually heavy rainfall (194).

In the Indian subcontinent, sporadic cases of melioidosis have been reported from Sri Lanka, India, Pakistan and Bangladesh (21;130;172;217). In Africa, the epidemiology of melioidosis remains uncertain and is hampered by a lack of diagnostic facilities. Melioidosis has been described in a goat in Chad and in pigs in Niger (89;179). Human melioidosis has also been reported from Uganda, Kenya and the Gambia (18;248).

1.2.2 Risk factors for melioidosis.

Risk factors for melioidosis have been evaluated by both case-control studies (220;222), and population based studies (69). Identified risk factors are summarized in table 1-2. An associated predisposition is present in 62% to 87% of patients with melioidosis (69;183;222).

Table 1-2. Risk factors for melioidosis.

Risk factor	Details
Male gender	Males predominate in all case series, the ratio of males to females ranging from 1.4:1 in Thailand (222), 3:1 in northern Australia (66), 3.2:1 in Malaysia (183), to 4.6:1 in Singapore (108).
Age	Age > 45 years is an independent risk factor in northern Australia (RR 4.0) (69), and incidence of melioidosis is highest in population aged 40-59 years in Thailand (RR 4.1) (222).
Aboriginal nationality	RR of 3.0 in northern Australia (69). This is assumed to be associated with environmental exposure associated with lifestyle.
Diabetes mellitus	60% of patients in Thailand (222), 58% of patients in Singapore (108), 38% of patients in Malaysia (183), and 37% of patients in northern Australia (66) are diabetic, mainly type II.
Chronic lung disease	RR of 4.3 in northern Australia (69). This underlying condition has not been identified as a risk factor in Thailand.
Chronic kidney disease	RR of 3.2 in northern Australia (69) and OR of 2.9 in Thailand (220). In Thailand, chronic kidney disease included renal calculi, which were found in 8% of patients (222).
Alcohol excess	RR of 2.1 in northern Australia (69). This was not reported in studies from Thailand (31;222), although 12% of melioidosis patients in Thailand had a history of heavy alcohol consumption (220).
Thalassemia	OR of 10.2 in Thailand (220), including beta-thalassemia/hemoglobin E and hemoglobin H disease.

1.2.3 Disease acquisition.

Melioidosis occurs as a result of exposure to environments containing *B. pseudomallei*. Acquisition occurs via inoculation, inhalation and ingestion. The major route of infection in farmers in northeast Thailand is likely to be contamination of existing wounds or new penetrating wounds with *B. pseudomallei* present in soil and water (222). Children infected with *B. pseudomallei* often have a history of repeated exposure to water and soil in rice fields (73). In Australia, aboriginals living in remote communities have frequent exposure to tropical soils and surface water through daily activities and hunting (69). Melioidosis in US helicopter crews and servicemen involved in the war with Vietnam is likely to represent infection via the respiratory route (90). An outbreak associated with contaminated drinking water (122) indicates that ingestion is an additional possible route, although this is probably rare. Near-drowning is also a known route of acquisition (222). This was highlighted by a cluster of melioidosis cases in PhangNga following the 2004 tsunami (47;55).

A case of mother-to-child transmission of *B. pseudomallei* has been reported (5). This involved a 31-year-old pregnant woman who was taking prednisolone for ulcerative colitis, which may have increased her susceptibility to infection. Identical *B. pseudomallei* isolates were obtained from both placenta and neonatal blood culture. Two cases of transmission of *B. pseudomallei* via breast milk have also been described (185). Both lactating mothers had mastitis. In one case, *B. pseudomallei* isolated from breast milk and from the infant were identical on PFGE. Possible sexual transmission has also been reported between a Vietnamese veteran with prostatitis and his wife (158). Person-to-person transmission may have occurred between a diabetic brother and sister living in endemic northeast Thailand (140), and between 2 siblings with cystic fibrosis in Australia (110). Suspected nosocomial melioidosis from hospital ground (15), contaminated of chlorhexidine-cetramide (Savlon) (181) and contaminated hand wash

detergent (94) have been reported. However, a direct cross-infection between hospital in-patients has not been reported in the published literature. Rare laboratory acquired infections have also been reported (102;196).

1.2.4 Prevention of melioidosis.

Protective gear, including waterproof shoes or boots and protective gloves are recommended for agricultural workers in endemic areas. This is particularly pertinent to individuals at high risk of melioidosis. However, these are often uncomfortable when worn for prolonged periods in the heat of the tropics and are rarely used. The efficacy of this advice has not been evaluated for the prevention of melioidosis. People with risk factors for melioidosis should stay indoors during periods of heavy wind and rain due to the potential for aerosolisation of *B. pseudomallei*.

There is currently no vaccine available against melioidosis, although several groups are exploring potential vaccination strategies (163;253). In a mouse model, CD4+ T cells can mediate vaccine-induced immunity to experimental melioidosis (107). Some protection has also been demonstrated in the mouse model following vaccination with *B. thailandensis* and other attenuated strains (119;120). However, it may prove difficult to develop a protective vaccination for individuals living in highly endemic areas who are already antibody positive for *B. pseudomallei*.

1.2.5 Reactivation of melioidosis after latent infection.

Reactivation of latent infection (which is normally taken to mean clinical features of melioidosis in an individual previously exposed to *B. pseudomallei* but with no history of a primary episode, as opposed to relapse which is recurrent features in a person who has responded to treatment for a primary episode of melioidosis), is well recognized and led to concerns regarding soldiers returned from Vietnam and travellers

exposed in endemic areas. The estimated number of potential 'cases' (defined as the presence of antibodies to *B. pseudomallei*) from the Vietnam War is around 250,000 cases (56), while only a number of reactivated infections have been reported; latent periods of 26, 29, and 62 years have been documented (50;157;164).

In melioidosis endemic areas, reactivation of latent disease is presumptively diagnosed in patients admitted to hospital for an unrelated illness who subsequently develop melioidosis during admission, and who were previously asymptomatic but had pre-existing abnormalities such as an abnormal chest radiograph involving the same lung region. A prospective study in Australia reported that only 3% of patients were considered to have reactivation of latent infection rather than recent acquisition (64).

It is unclear whether high antibody titers in healthy individuals reflect quiescent foci. There is no evidence for a *B. pseudomallei* carrier state in humans, with recovery of the organism from a clinical specimen indicating active disease (129). The strong seasonal association of this disease and short incubation period suggests that primary disease occurs as a new infection rather than seasonal activation of a persistent focus. The other, less likely possibility is the seasonal development of underlying risk factors causing immune dysfunction, followed by seasonal activation.

1.2.6 Clinical manifestation of melioidosis.

1.2.6.1 Seasonal incidence of melioidosis.

Melioidosis is seasonal in the tropics where most cases present during the rainy season, which occurs between June and November in Thailand (222) and November to May in Australia (64). This can be explained by increased contact with the organism. Rice farmers plant at the start of the monsoon and work in flooded rice paddies until harvest. Thai farmers rarely wear protective footwear and their feet often show signs of repeated trauma and injuries. Extreme weather may be associated with a shift in the

mode of acquisition of infection. Aerosols are created during heavy rain, and this may result in repeated inhalation of the organism. Heavy rainfall and winds consistently result in a shift towards more pneumonia in patients presenting with melioidosis in Northern Australia (68).

1.2.6.2 Incubation period.

The period between *B. pseudomallei* exposure and onset of clinical features of melioidosis is highly variable and often difficult to define. A history of a specific inoculation event is often absent. In one study, 25% of cases reported a specific inoculation event from which an incubation period of 1-21 days (mean 9 days) was derived (67). The incubation period following aspiration may be very short, probably because the inoculum may be large and organisms gain direct access to a deep site (47). The incubation period may also be very prolonged (157), the maximum recorded being 62 years (164).

1.2.6.3 Duration of symptoms.

The time from onset of disease to clinical presentation is highly variable. In northeast Thailand, around a third of patients have symptoms for less than 7 days, one half report being unwell for 7-28 days, and the remainder have symptoms for more than 28 days (266). In Northern Australia, 13% of patients presenting for the first time had symptoms for more than two months (67).

1.2.6.4 Clinical syndromes.

Manifestations of disease are extremely broad and range from an acute fulminant bacteraemia to a chronic debilitating localized infection. Melioidosis is a great mimicker, and it is often impossible on clinical grounds to differentiate between

meliodosis and other acute and chronic bacterial infections, including tuberculosis. The majority of adult patients develop an acute septicaemia associated with bacterial dissemination that most commonly manifests as pneumonia and/or hepatosplenic abscesses. Multiple organ involvement, and the development of additional organ involvement following presentation are also common (66).

1.2.6.4.1 Bacteraemia.

Bacteraemia is found in around half of melioidosis patients in prospective studies: 46% in Australia (66), 59% in Malaysia (183), and 60% in Thailand (220). The lung is the commonest site of infection associated with bacteraemic melioidosis. Bacteraemia alone without localized organ involvement is also found (183). Septic shock with a brief incubation period is common and is frequently complicated by the development of irreversible organ damage and the multiple organ dysfunction syndrome. The mortality of melioidosis with septic shock varies from 60% in Singapore (22), 86% in Australia (66), and 90% in Thailand (38).

1.2.6.4.2 Lung involvement.

The lung is the most commonly affected organ and is involved in approximately half of all cases, either presenting with primary pneumonia or lung abscess, or secondary to bacteraemic spread (blood-borne pneumonia). Sputum is often purulent but rarely blood-stained. Large or peripheral lung abscesses may rupture into the pleural space to cause thoracic empyema. The common radiographic patterns for acute pneumonia are localized patchy alveolar infiltrate, bilateral diffuse patchy alveolar infiltration or multiple nodular lesions consistent with haematogenous spread (188). Upper-lobe involvement with early cavitation and rapid progression are common. Upper lobe infection in a patient with a subacute or chronic presentation can be difficult

to distinguish from pulmonary tuberculosis on the basis of clinical features and radiological changes.

1.2.6.4.3 Liver and spleen involvement.

Solitary or multiple abscesses may develop in the liver and/or spleen. Hepatosplenic abscess is reported to be present in 28% of melioidosis patients in Thailand (201). This is the most common cause of pyogenic liver and splenic abscess in northeast Thailand (195;244). By contrast, hepatosplenic abscess was found in only 6% of melioidosis patients in Australia (66). Multiple abscesses are more common than a solitary abscess in both organs. The finding of a “Swiss cheese” appearance on ultrasonogram and “honeycomb” appearance on computed tomography (CT) scan are characteristic for melioidosis (11). More than half of patients with hepatosplenic abscess lack abdominal pain and abdominal tenderness (82). Spread of infection from a splenic abscess to the pleura, lung, adjacent stomach, liver, diaphragm and rib have been reported (226).

1.2.6.4.4 Genitourinary tract involvement.

Genitourinary infection is common in Australia, with prostatic abscesses occurring in 18% of male patients (66). A common clinical presentation of prostatic abscess is an obstructed urinary tract leading to urinary retention (225). On transrectal ultrasonogram, prostatic abscesses in patients with melioidosis are reported to appear alone or with hypo-echoic areas with internal septation, a thickened wall and surrounding multiple small daughter abscesses. Renal abscesses are often associated with calculi and urinary tract infection. Infection involving the urinary tract is present in at least one quarter of Thai patients based on a urine culture positive for *B.*

pseudomallei, although only a quarter of positive cases had urinary symptoms such as urinary frequency, dysuria, haematuria, or flank or back pain (148).

1.2.6.4.5 Skin and soft tissue involvement.

Superficial pustules, subcutaneous abscesses and pyomyositis are common manifestations of melioidosis and may be the source of systemic infection, or may result from haematogenous or local spread. Skin and soft tissue infections are found in 24% of melioidosis patients in Thailand (46) and 17% in Australia (66). Infection may run an aggressive course similar to that seen for necrotizing fasciitis caused by other organisms (252).

1.2.6.4.6 Neurological involvement.

Neurological melioidosis characterized by brainstem encephalitis and flaccid paraparesis is defined in 4% of melioidosis cases in northern Australia (33). Prominent features on presentation are unilateral limb weakness, predominant cerebellar signs, mixed cerebellar and brainstem features with peripheral weakness and flaccid paraparesis (65). Peripheral motor weakness may mimic Guillain-Barre syndrome (117) and unilateral limb weakness may mimic a stroke (137). Focal suppurative infections involving the central nervous system with or without meningitis have been reported. CNS infections occur in around 1.5% of melioidosis patients in Thailand, while the syndrome of meningoencephalitis has not been defined in this setting (147). Early brain CT scan may be normal, but MRI often shows dramatic changes (65;147).

1.2.6.4.7 Osteoarticular involvement.

Osteomyelitis and septic arthritis due to melioidosis have been reported and may be difficult to differentiate from other causes of infection, except that the systemic

features of illness may be more prominent. Osteoarticular involvement was found in 17% of melioidosis patients in Thailand (46) and 4% in Australia (66;177). The knee is the most common site of septic arthritis (260). Osteomyelitis is often secondary to infection of another organ; however, localized osteomyelitis from *B. pseudomallei* has been reported (219).

1.2.6.4.8 Parotid gland involvement.

A unique syndrome of acute suppurative parotitis is present in around one-third of paediatric cases in Thailand (73), but this is uncommon in Thai adults and has been reported in one case from Australia (88). Infection is bilateral in 10% of patients, and may be complicated by rupture into the auditory canal, facial nerve palsy and necrotizing fasciitis (73;146).

1.2.6.4.9 Other organ involvement.

Infections involving many other sites have been described, including lymphadenitis (49), mycotic aneurysm (150), adrenal gland abscess (142), mediastinal infection (189), pyoperdicarditis (145;153), deep neck abscess (213), acute otitis media (257), sinusitis (146), corneal ulcers (204), orbital cellulitis (258), breast abscess (143), and scrotal abscess (125).

1.2.7 Diagnosis of melioidosis.

Melioidosis should be suspected in any severely ill febrile patient with an underlying predisposing condition who lives in, or has travelled from a melioidosis-endemic area. Evidence of pneumonia, abscess formation in the liver and spleen on ultrasound examination, soft tissue abscess and septic arthritis should be sought. The

presence of a splenic abscess in a person living in an endemic area is highly suggestive of *B. pseudomallei* infection.

The definitive diagnosis of melioidosis requires positive bacterial culture and identification. Microbiological culture should be performed on all available specimens including blood, urine, throat swab, respiratory secretions, pus and swabs from surface wounds. As *B. pseudomallei* is not a member of the normal flora and is not carried asymptotically, a positive culture from any site is highly suggestive of infection. Because the organism can become disseminated in the host, the site of culture positivity may not necessarily reflect the major site of infection. For example, a positive throat swab may occur in a patient with a deep site abscess, and positive urine cultures may reflect spill over from the bloodstream as well as renal involvement.

The polymerase chain reaction (PCR) has been evaluated for the diagnosis of melioidosis but is not in routine use. A prospective unblinded evaluation in Australia that used primers targeting a region of the TTSS1 gene cluster demonstrated a sensitivity of 91% compared with standard culture for the diagnosis of melioidosis (160). However, a prospective blinded evaluation in Thailand using PCR primers targeting a region of the 16s rRNA gene showed a lower diagnostic sensitivity of 61% (25). Comparison of sensitivity of the assays using TTSS1 and 16s rRNA primers in the same population showed that the TTSS1 assay was inferior to 16s rRNA assay (23).

Serological diagnosis is possible in patients living in a non-endemic area who have not emigrated from an area of endemicity, but serology is not specific in melioidosis-endemic regions because the rate of background positivity in the healthy population is high (40). This is discussed in more detail in section 1.2.7.2 below.

Clinical diagnosis is an imperfect strategy but is sometimes necessary when cultures are suspected to be falsely negative, or in settings where culture is not available.

1.2.7.1 Laboratory identification of *B. pseudomallei*.

B. pseudomallei grows on most routine laboratory media (14). The organism exhibits considerable inter-strain and medium dependent variability in colonial morphology (78), and the wrinkled appearance of older colonies has occasionally resulted in their being overlooked as contaminants. In specimens containing a mixed normal bacterial flora, *B. pseudomallei* may be overgrown by commensals and consequently overlooked. Several selective media have been developed to overcome this problem, the most widely used being Ashdown's selective agar. This is a simple agar containing crystal-violet, glycerol and gentamicin. Mature colonies often take on a wrinkled appearance and take up crystal violet dye from the medium after several days of incubation, but some strains do not appear wrinkled. A recent study described and characterised seven colony morphology types for *B. pseudomallei* on Ashdown's agar, type I (the wrinkled type) being the most common type found in clinical samples and the environment (24). Selective broth for the isolation of *B. pseudomallei* from clinical samples containing normal flora is also required to prevent overgrowth by other species (251). Use of the selective broths significantly increases the frequency of isolation of *B. pseudomallei* over direct plating on Ashdown's agar alone (265).

Laboratory identification of *B. pseudomallei* is a challenge to laboratories that are unfamiliar with the bacterium. The organism may be overlooked as "*Pseudomonas* spp.". Biochemical markers of *B. pseudomallei* include a positive oxidase reaction, production of gas from nitrate, arginine dihydrolase and gelatinase activities and oxidation of a wide variety of carbohydrates. Common bacterial identification systems including the API20NE (bioMerieux), RapID (Remel) and the Vitek automated system are of variable reliability for the identification of *B. pseudomallei* (96). *B. pseudomallei* can be misidentified as *B. cepacia*, *Pseudomonas aeruginosa*, *Chromobacterium violaceum* and a range of other species. Latex agglutination using a specific

monoclonal antibody to EPS is useful for the rapid identification of *B. pseudomallei* and differentiation from *B. thailandensis* (positive for *B. pseudomallei* and negative for *B. thailandensis*) (9). This assay has 100% accuracy for the rapid identification of *B. pseudomallei* colonies growing on solid agar (81), but uses an in-house reagent which is not commercially available.

Direct immunofluorescent microscopy of infected sputum, urine, or pus is 98% specific and 70% sensitive compared with culture, and allows a diagnosis to be made within 10 minutes (249). Again, this uses an in-house reagent. Several PCR based techniques have been developed for the identification of *B. pseudomallei* (23;25;160), although these are not in routine use in the diagnostic laboratory setting.

1.2.7.2 Serological tests for *B. pseudomallei*.

Most healthy individuals (>80%) living in northeast Thailand develop antibodies to *B. pseudomallei* during childhood and some healthy individuals have very high titres (129;264). As a result, serological diagnosis has low specificity in melioidosis-endemic regions. Interpretation of serology is more straightforward in the non-endemic setting provided a knowledge of travel history and place of normal residence is available. A rising antibody titre to *B. pseudomallei* in paired sera taken from an individual who does not normally reside in an endemic area supports the diagnosis of melioidosis in the presence of clinical features of disease.

Numerous reports have been published describing the use of IHA to define seropositivity in healthy populations and patients with culture-proven or suspected melioidosis. The diagnostic cut-off used to define a positive result has varied between studies; $\geq 1:10$ has been used to define seroconversion in Thailand (32;264), and titres of $\geq 1:16$, $1:40$ and $1:160$ have all been used to define a diagnostic titre (12;13;108;261;272). The IHA test has poor diagnostic specificity in areas of high

endemicity (12;261). Increasing the IHA cut-off leads to a rise in specificity but loss of sensitivity (166). The IHA is more useful in Singapore, where disease is sporadic and background seropositivity is <1% (108;272). Using a cut-off titre of 1:16, less than 1% of the asymptomatic population had a positive titre, while 20 patients with melioidosis had titres of 1:16 or greater (108;272).

Several other tests have been described, including an indirect immunofluorescence assay (IFA) (133;154;239), enzyme-linked immunosorbent assay (ELISA) using culture filtrate or affinity purified antigen (16;241), and dot immunoassay (197;259). Data on their clinical utility is sparse and none of these tests have replaced IHA. An immunochromatographic test kit has been developed for the rapid detection of IgG and IgM antibodies to *B. pseudomallei*, and this has been evaluated in Thailand and Australia (40;54). Based on results for IgG, this kit represents an alternative to the IHA but the product will not be released onto the market.

1.2.8 Antimicrobial susceptibility of *B. pseudomallei*.

B. pseudomallei is intrinsically resistant to many antibiotics, including first, second and third-generation cephalosporins, aminoglycosides, penicillins and polymyxin (159;232). Inherent resistance to ampicillin and broad- and expanded-spectrum cephalosporins is due to the production of beta-lactamases. This is a weakly inducible, membrane associated chromosomal cephalosporinase which is strongly active against carbenicillin, cefotaxime and cefuroxime, and inactivated by clavulanic acid (149). The beta-lactamase gene *PenA* has conserved motifs typical of class A beta-lactamase and is related to *PenA* (in *B. cepacia*) and *Blal* (in *Yersinia enterocolitica*) lineages (44). Macrolide and aminoglycoside resistance is mediated via *AmrAB-OprA*, a multidrug efflux pump which is specific for both macrolides and aminoglycoside

antibiotics (161). Resistance to ceftazidime or amoxicillin with clavulanic acid (AMC) is rare, although the development of resistance to both drugs during treatment has been reported (75). Carbapenem resistance has not been reported in the published literature. Trimethoprim-sulfamethoxazole (TMP-SMX) susceptibility testing using the disc diffusion method grossly overestimates the rate of resistance (60 to 70%); the E test defines the rate of resistance to be around 13% in Thailand (263), and at the much lower rate of 2% in Australia (174).

B. pseudomallei is usually susceptible to TMP-SMX, chloramphenicol, the tetracyclines, ceftazidime, carbapenems, AMC, piperacillin, piperacillin-tazobactam, ticarcillin-clavulanate, ampicillin-sulbactam, and carumonam (209). Carbapenem antibiotics have the greatest activity *in vitro*, while fluoroquinolones have poor activity *in vitro* (77;232), and had very low efficacy in a clinical trial (30). Rare clinical isolates are susceptible to gentamicin and macrolides, occurring in the order of approximately 1 in every 1,000 isolates (202). The beta-lactams and ciprofloxacin are bactericidal *in vitro*, whereas the agents conventionally used to treat melioidosis during the oral phase of treatment (TMP-SMX plus doxycycline) have bacteriostatic activity only (77).

1.2.9 Treatment of melioidosis.

Empirical antimicrobial treatment of patients with suspected melioidosis is associated with a significant reduction in mortality rate (28). Cefotaxime and ceftriaxone are both less active than ceftazidime, and should not be used for empirical treatment of suspected melioidosis.

Treatment is divided into acute intravenous and oral eradication phases. Initial intravenous therapy is required for at least 10 to 14 days, or until clinical response is seen (whichever is the longer). This duration may be exceeded in critically ill patients, those with extensive pulmonary disease, deep-seated collections or organ abscesses.

Intravenous therapy for 4 to 6 weeks may be required for patients with osteomyelitis, septic arthritis or neurological melioidosis (147). After completing the initial parenteral phase, oral eradication treatment is required for at least 12 to 20 weeks. Patients with mild localized disease are occasionally treated with oral antimicrobial drugs alone.

1.2.9.1 Initial parenteral treatment.

Ceftazidime is the treatment of choice for melioidosis. This is based on the most important clinical melioidosis trial showing a 50% reduction in mortality from 74% to 37% associated with the use of ceftazidime compared with a combination of doxycycline, chloramphenicol and TMP-SMX (255). A reduction in mortality of a similar magnitude was reported for ceftazidime plus TMP-SMX compared with a combination of doxycycline, chloramphenicol and TMP-SMX (208). Ceftazidime, with or without TMP-SMX, became the standard of care for the initial therapy of patients in Thailand thereafter. Studies comparing cefoperazone-sulbactam plus TMP-SMX versus ceftazidime plus TMP-SMX showed equivalence for the two arms (43;231). Uncertainty over whether TMP-SMX is necessary during initial treatment has since been addressed by a randomised trial of ceftazidime alone versus ceftazidime plus TMP-SMX (46). The addition of TMP-SMX to ceftazidime therapy during initial treatment of severe melioidosis does not reduce the acute mortality rate (46).

The carbapenem antibiotics have several theoretical advantages over ceftazidime based on *in vitro* susceptibility data. However, a trial comparing imipenem and ceftazidime reported no difference in survival overall, or after 48 hours (201). There was a higher but non-significant difference in treatment failure after 48 hours for the ceftazidime group (201). This trial was stopped prematurely due to interruption of imipenem supply, and as a result was underpowered. A retrospective review of the use of meropenem in Australia concluded that it may be associated with improved

outcomes in patients with severe sepsis associated with melioidosis, although this study was confounded by several concurrent changes in intensive care management (35). A prospective multicentre evaluation of meropenem versus ceftazidime is currently being performed in northeast Thailand.

AMC is the treatment of choice for pregnant women. A clinical study comparing AMC and ceftazidime demonstrated a similar mortality rate, but 4 of 75 (5%) surviving patients in the ceftazidime group compared with 16 of 69 (23%) surviving patients in the AMC group were switched to an alternate regimen because of an unsatisfactory clinical response after >72 hours of treatment (224).

1.2.9.2 Route of parenteral ceftazidime: bolus versus infusion.

In a study of the pharmacokinetic and pharmacodynamic effects of continuous infusion of ceftazidime vs intermittent bolus dosing, 34 patients suspected to have septicaemic melioidosis (of whom 20 had cultures positive for *B. pseudomallei*) were randomised to receive ceftazidime by either bolus injection or by constant infusion following a priming dose (6). Simulations based on these data and the ceftazidime sensitivity of the *B. pseudomallei* isolates indicated that administration by constant infusion could provide adequate dosing with a significant dose reduction and cost saving (6). Ceftazidime infusions are now being used in Darwin, Australia to facilitate home intravenous therapy after discharge from hospital (118). The cost of peripherally inserted central catheter lines and infusions, together with the cost and infrastructure required to support home IV therapy prohibits their use in most endemic regions of Asia.

1.2.9.3 Monitoring of antimicrobial levels during treatment.

In a retrospective evaluation of the relationship between serum bactericidal and inhibitory titers and treatment outcome in 195 adult Thai patients with severe melioidosis, pre- and 1 hour post-dose serum samples were collected after 48-72 hours of therapy, and serum inhibitory and bactericidal titrations were determined (200). Drug regimens included ceftazidime (52% of patients), AMC (24%), imipenem (11%) or the conventional four-drug combination (11%). Overall mortality was 26% and outcome was not influenced by either inhibitory or bactericidal titers (200). Thus, these measures do not appear to have a role in the management of melioidosis. A time-kill study of 10 *B. pseudomallei* isolates using a range of concentrations demonstrated that a ceftazidime concentration of eight times the MIC yielded an optimal bactericidal effect (210), but the relevance of this observation for clinical practice is not defined.

1.2.9.4 Adjuvant therapy.

Granulocyte colony-stimulating factor (G-CSF) was adopted for use in patients with septic shock due to melioidosis at the Royal Darwin Hospital, northern Australia in December 1998. This was associated with a decrease in mortality from 95% to 10% (41). Risk factors, the duration of illness before presentation and severity of illness were similar in patients before and after G-CSF introduction, but improvements in intensive care management also occurred during the study period. A randomised placebo-controlled trial of G-CSF for severe melioidosis performed at Sappasithiprasong Hospital failed to show a difference in mortality (38). A placebo-controlled trial of an intravenous PAF receptor antagonist (lexipafant) in 131 adult Thai patients with suspected severe sepsis included 36 patients with melioidosis; no benefit was detected (223). Other interventions shown to be of benefit in critically ill septic patients such as goal-directed therapy (192), low tidal volume ventilation (3), intensive

glycaemic control (243), and activated protein C (19) have not been evaluated in patients with melioidosis.

1.2.9.5 Supportive treatment and other management of patients with melioidosis.

Initial intensive care management of severe melioidosis is similar to that of any severe sepsis. Patients need to be resuscitated with correction of electrolyte imbalance and adequate intravenous fluid, since hypovolemia is common in the acute phase. Septic arthritis requires joint washout, and large accessible abscesses require drainage. For suppurative parotitis, surgical drainage is required to avoid lower motor neuron seventh-nerve palsy. Standard of care and availability of ICU in different geographical regions is likely to be an important contributor to the marked difference in outcome between Australia and Thailand.

Routine laboratory tests should be performed to detect the onset of acute renal failure, abnormal liver function tests and anaemia, all of which are well recognized during severe melioidosis. Arterial blood gases should be taken in patients with severe sepsis, lung involvement and/or any evidence of respiratory impairment. Serum C-reactive protein levels (CRP) do not appear to aid patient management, based on a review of CRP in 175 patients which found that admission CRP may be normal or only mildly elevated, including patients with severe sepsis, fatal cases, and in recurrent melioidosis (39).

Chest radiographs should be taken in all patients with suspected melioidosis. Common radiographic patterns include diffuse interstitial shadowing considered consistent with blood borne spread of infection, localized patchy alveolar infiltrate, focal, multifocal or lobar consolidation, pleural effusion and upper-lobe involvement which may include cavitation (188). Patients may develop empyema and/or lung abscess. Abdominal ultrasound or CT scan is required to detect liver and/or splenic

abscesses. Clinical evidence of prostatic involvement requires appropriate imaging (trans-rectal ultrasound or CT scan) (225).

1.2.9.6 Oral eradication treatment.

Oral therapy after completion of parenteral treatment is required to eradicate the organism and prevent relapse of the disease. TMP-SMX based regimens are the treatment of choice. TMP-SMX is used in Australia while TMP-SMX plus doxycycline is generally used in Thailand. The three drug regimen used in Thailand replaces a previous four drug regimen that included chloramphenicol, which was discontinued following a prospective randomised controlled trial that showed no difference in the rate of recurrence between the three-drug and four-drug regimens (26). Doxycycline monotherapy is not appropriate since this was shown to be associated with a significantly higher culture-confirmed relapse and clinical treatment failure compared with the four-drug regimen (27).

AMC is the oral treatment of choice for pregnant women, young children, and for those with hypersensitivity reactions to TMP-SMX or doxycycline. In a trial of AMC versus the four-drug combination, the rate of relapse was higher for the AMC group (16% versus 4%) but only half of the patients completed the intended 20 weeks of therapy. Poor compliance proved the most significant risk factor for subsequent relapse (184). Uncertainty remains as to whether 20 weeks of AMC is less effective than combination treatment. AMC does not pass through the blood brain barrier and should not be used in patients with neurological melioidosis (147).

Fluoroquinolones are not recommended for the oral treatment of melioidosis. A comparison of ciprofloxacin and azithromycin for 12 weeks versus TMP-SMX and doxycycline for 20 weeks demonstrated a relapse rate of 22% and 3%, respectively (42). The relative contribution of differences in treatment duration in the two arms to

the rate of relapse is not known. However, ciprofloxacin or ofloxacin given for a median of 15 weeks to treat 57 adult patients with melioidosis was associated with an unacceptably high failure rate of 29% (30).

Options for treatment of patients with neurological melioidosis and intolerance to TMP-SMX or infected with a strain that is resistant to TMP-SMX are very limited. Prolonged parenteral therapy is impossible in resource-poor regions, where probably the best option is chloramphenicol plus doxycycline (147). New agents for the oral eradication of melioidosis are much needed. *B pseudomallei* is susceptible to some of the new oral cephalosporins including cefixime and cefetamet (77). These drugs should be further evaluated for safety and efficacy in the oral treatment of melioidosis.

1.2.9.7 Summary of recommendations for antimicrobial therapy of melioidosis.

Initial parenteral therapy is given for at least 10 to 14 days, until there is clear evidence of clinical improvement. Ceftazidime or a carbapenem drug is the treatment of choice (table 1-3). Ceftazidime is the drug of choice in Thailand, with a switch to a carbapenem antibiotic if the patient fails treatment. Parenteral treatment at the Royal Darwin Hospital is ceftazidime, or meropenem plus G-CSF if the patient has septic shock (41). The addition of TMP-SMX to ceftazidime or meropenem during the initial intensive therapy phase has been phased out in Darwin (62), although this drug is still used to treat patients with neurological (147) or prostatic melioidosis (171). Alternative parenteral treatment for pregnant women in Thailand is AMC. Treatment duration longer than 14 days may be required in critically ill patients, those with extensive pulmonary disease, deep-seated collections or organ abscesses. Patients with large abscesses or empyema often have fluctuating fevers for more than 1 month. Four to six weeks of parenteral treatment may be required for patients with osteomyelitis, septic arthritis or neurological melioidosis (147). Enlargement of an existing abscess, the

appearance of new abscesses or seeding to a joint are common during the first week of parenteral treatment and should not necessarily be assumed to be a sign of treatment failure.

Oral eradication treatment for at least 12-20 weeks consists of TMP-SMX alone (Australia) or together with doxycycline (adults in Thailand) (table 1-3). Close follow-up and monitoring of drug adherence are important. Currie *et al.* reported that the recurrence rate associated with TMP-SMX relates almost exclusively to non-compliant patients (64). First-line oral treatment for pregnant women and children is AMC; this is also an alternative for adults who cannot tolerate TMP-SMX. AMC does not pass through blood brain barrier and should not be used in patients with neurological melioidosis (147).

Table 1-3. Antimicrobial treatment of melioidosis.

Sappasithprasong Hospital	Royal Darwin Hospital
<i>a. Initial parenteral therapy</i>	
<p>Ceftazidime 40 mg/kg (up to 2 g) every 8 hours, or meropenem 25 mg/kg (up to 1 g) every 8 hours, or imipenem 20 mg/kg (up to 1 g) every 8 hours, or AMC at 20/5 mg/kg (up to 1.2 g) every 4 hours for pregnant woman.</p> <p>Duration at least 10 to 14 days or until clear clinical improvement, which ever is the longer.</p>	<p>Ceftazidime 50 mg/kg (up to 2 g) every 6 hours, or meropenem 25 mg/kg (up to 1 g) every 8 hours plus G-CSF (filgrastin) 300 µg for 10 days if patient has septic shock.</p> <p>Duration of therapy at least 14 days, and longer (4 to 8 weeks) for deep-seated infection.</p>
<i>b. Oral eradication therapy</i>	
<p>Adults: TMP-SMX 5/25 mg/kg (up to 320/1,600 mg) respectively every 12 hours, ^a plus doxycycline 2 mg/kg (up to 100mg) every 12 hours.</p> <p>Children ≤8 years and pregnant women: AMC 20/5 mg/kg (up to 1500/375 mg) respectively every 8 hours. ^b</p> <p>Duration at least 12-20 weeks.</p>	<p>TMP-SMX 8/40 mg/kg (up to 320/1,600 mg) every 12 hours.</p> <p>Duration at least 3-6 months.</p>

^a Equivalent to 3 x 480mg TMP-SMX tablets every 12 hours for a patient <60kg, and 4 x 480mg TMP-SMX tablets every 12 hours for a patient >60kg

^b Equivalent to 2 x 625mg AMC tablets every 8 hours for an adult patient <60kg, and 3 x 625 mg AMC tablets every 8 hours for an adult patient >60kg

1.3 Recurrent melioidosis.

The single most important complication for patients who survive their first episode of melioidosis is recurrence, which may occur despite prolonged oral eradication antimicrobial treatment. Recurrent melioidosis is defined as the development of new symptoms and signs of infection after responding to therapy for the primary episode. Mortality from recurrence has been reported to be as high as that for primary infection (29). To date, recurrence has been considered to be largely synonymous with relapse caused by the re-emergence of a quiescent focus, rather than re-infection with a new strain.

1.3.1 Background of recurrent melioidosis.

In 1975, Mays and Ricketts described a patient with melioidosis who developed a sputum culture positive for *B. pseudomallei*, 3 weeks after completing three and a half months treatment with oral tetracycline followed by a further month of chloramphenicol (157). A case series in North Queensland Australia between 1979 and 1981 described recurrent melioidosis in 3 of 14 (21%) patients, all three of whom drank alcohol to excess and were poorly compliant with the oral antimicrobial medication (104).

There were no guidelines for oral eradication treatment at that time. In 1967, using experience from the Vietnam war Dr Deller recommended treatment with a three drug combination of 12 grams of chloramphenicol, 3 grams of kanamycin and 6 grams of novobiocin for the treatment of acute melioidosis, or 1 gram of tetracycline daily for chronic melioidosis (1). The necessity of oral eradication treatment after recovery from acute melioidosis was not defined. Failure of tetracycline monotherapy in chronic melioidosis was also reported in 1971 (228). Susceptibility testing showed that the strain had developed resistance to tetracycline after three weeks of treatment.

Chloramphenicol was found to be effective but was associated with adverse drug events (178). *B. pseudomallei* was also found to be susceptible to TMP-SMX and successful treatment with this drug combination was reported (93;126).

Various drug combinations were subsequently recommended for oral eradication treatment with the aim of reducing the rate of recurrence. In 1981, Rode and Webling suggested a four-drug combination of tetracycline or doxycycline, chloramphenicol, and TMP-SMX (193), and Puthucheary *et al.* recommended a combination of tetracycline and chloramphenicol for at least 6 months to prevent relapse (182). Chloramphenicol in the four-drug regimen was normally prescribed only for the first month (157). The four-drug regimen was widely adopted in Thailand where it continued to be used until the publication in 2005 of a trial indicating that three drug combination of doxycycline and TMP-SMX was as effective as the four drug combination (26). The recommended duration of oral treatment varied from 3 to 6 months (104).

A prospective study of 118 melioidosis patients in Thailand from 1986 to 1991 found that 27 (23%) had culture-proven recurrent melioidosis (29). The median time from start of oral eradication treatment to recurrence was 21 weeks (range 1 - 290 weeks). Bacteraemia recurred in 44% of patients, and 27% of the patients died. Poor compliance was found during interview with three patients with recurrence. Recurrent strains were found to be less susceptible to the antibiotics used in three cases. The recurrence rate was also found to be very high (19%) in a retrospective review from Pahang, Malaysia (115).

In a 10-year prospective study of melioidosis in the Northern Territory of Australia between 1989 and 1999, 27 of 222 (13%) patients surviving the acute phase developed recurrence (64). The mean time to recurrence was 32 weeks. Half of the

recurrent patients had adhered poorly with oral antibiotic therapy, and 10 represented failure of eradication with doxycycline monotherapy.

1.3.2 Clinical trials of oral eradication treatment to prevent recurrence.

A four-drug combination of chloramphenicol, doxycycline and TMP-SMX was the recommended oral eradication treatment for melioidosis in Thailand from 1981 (193); however, adverse drug reactions from these four drugs are extremely high. The use of high dose chloramphenicol for long periods is associated with haematopoietic suppression and the induction of aplastic anaemia. Allergic reactions to sulphonamides are potentially severe and the tetracyclines cannot be used in pregnancy, childhood and renal failure. A large number of tablets must be taken each day, which reduces the chances of good compliance. This led to the evaluation of alternatives, as summarized in table 1-4.

A pilot study of AMC monotherapy was conducted between June 1986 and August 1989 at Sappasithiprasong Hospital, northeast Thailand (221). Intended duration of therapy was a minimum of 8 weeks. AMC was given to 46 patients for a median duration of 7.5 weeks. Culture-proven recurrent melioidosis occurred in 13 of 46 (28%) cases after a median follow-up of 6 months. The drug was generally tolerated despite the high doses used and thereafter was considered an alternative for children, pregnant women, infection with a *B. pseudomallei* strain resistant to TMP-SMX, and for patients who were allergic to any drug within the four-drug regimen.

An open randomised controlled trial comparing AMC with the four-drug regimen was conducted between June 1989 and October 1992 (184). A total of 101 patients who recovered from acute melioidosis were enrolled into the study. Because of the high recurrence rate in the AMC study described above (221), total duration of treatment was extended to 20 weeks. Recurrent melioidosis occurred in 2 of 52 (4%)

patients in the oral four-drug regimen group and 8 of 49 (16%) patients in the oral AMC group (adjusted RR 0.4; 95% confidence interval (CI), 0.2-1.2) after a median follow-up of 18 months. The study suggested that AMC is safer and better tolerated, but may be less effective than the oral four-drug regimen. The minimum duration of oral treatment was defined thereafter as 12 to 20 weeks.

Although the fluoroquinolones have intermediate activity against *B. pseudomallei* *in vitro* in terms of minimum inhibitory concentration, efficacy in melioidosis is poor. Oral ciprofloxacin or ofloxacin monotherapy was evaluated in a study conducted between June 1991 and March 1995 at Sappasithiprasong Hospital (30). A total of 57 patients were enrolled into the study and 13 treatment failures (29%) occurred; of these, 5 failures represented treatment failure of the primary episode, and 8 had recurrence after an initial response. In one patient, fluoroquinolone resistance developed within one week of starting treatment. Effectiveness of a fluoroquinolone-based regimen was evaluated again in an open RCT conducted between August 1997 and July 1998 (42). A combination of ciprofloxacin and azithromycin was compared with TMP-SMX plus doxycycline. Of 65 patients enrolled, 7 of 32 (22%) patients in the ciprofloxacin arm and 1 of 33 (3%) patients in three-drug arm had recurrent melioidosis. As a result of these two studies, fluoroquinolones are now reserved as third-line drugs for patients who are either intolerant to other available antibiotics or who are infected with multi-resistant organisms.

The four-drug regimen has a high risk of toxicity and there is *in vitro* evidence of antagonism between the component drugs (76). TMP-SMX has been reported to antagonize the effect of chloramphenicol and doxycycline *in vitro*. Based on evidence of successful treatment with doxycycline monotherapy in Australia during an outbreak between 1990 and 1991 (63), an open randomised controlled trial comparing doxycycline alone with the four-drug regimen was conducted between October 1994

and August 1997 (27). Of 87 patients enrolled, 1 of 44 (2%) patients randomised to the four-drug regimen and 11 of 43 (26%) patients randomised to doxycycline monotherapy had recurrent infection (HR 12.0; 95%CI, 1.4-100.8). Development of doxycycline resistance was found in one patient with recurrence who was treated with doxycycline monotherapy. This study showed that doxycycline monotherapy cannot be recommended.

Chloramphenicol can cause serious toxicity and is associated with a high rate of adverse events, including gastrointestinal intolerance and allergic reaction, so the idea of removing this from the treatment protocol was a popular one (42;66). An open randomised controlled trial comparing four-drugs with three-drugs (minus chloramphenicol) was conducted between July 1998 and October 2002 (26). There was no difference in the recurrence rate, but the three-drug regimen was associated with a much lower rate of side effects (19% vs 36%). The duration of oral therapy was significantly associated with recurrent infection. Patients receiving treatment for less than 12 weeks had a 5.7 fold increase of recurrence or death. The three-drug regimen is now the standard treatment for oral eradication of melioidosis in Thailand.

As doxycycline monotherapy is ineffective as an oral eradication treatment (27) and TMP-SMX monotherapy has been used successfully in Australia (64), the role of doxycycline in the three-drug regimen has been questioned. A double-blind randomised controlled trial comparing the three-drug regimen with TMP-SMX monotherapy is now being conducted in Thailand (ISRCTN86140460). This study aims to enroll 635 patients who will complete a minimum of 1 year follow-up.

Table 1-4. Summary of studies evaluating oral antibiotic treatment of melioidosis.

Year of publication	Type of study	Oral antimicrobial treatment	Number of patients	Number of patients with recurrence	Conclusion of study	Ref.
1991	Pilot	AMC	46	13 (28%)	AMC is an alternative regimen for oral eradication treatment.	(221)
1993	Review	-	118	27 (23%)	Review of risk factors associated with recurrent melioidosis.	(29)
1995	RCT	Four-drug regimen versus AMC	52 49	2 (4%) 8 (16%)	AMC is safer but less effective. Duration of treatment extended to 12 to 20 weeks.	(184)
1997	Pilot	Ciprofloxacin or ofloxacin	57	8 (14%)	Fluoroquinolones cannot be recommended for oral treatment.	(30)

1999	RCT	Four-drug regimen versus doxycycline monotherapy	44 43	1 (2%) 11 (26%)	Doxycycline cannot be recommended for oral treatment.	(27)
2000	Review	-	207	27 (13%)	Review of manifestations of recurrent melioidosis in Australia	(64)
2001	RCT	Three-drug regimen versus ciprofloxacin and azithromycin	33 32	1 (3%) 7 (22%)	Ciprofloxacin based regimen is inferior	(42)
2005	RCT	Three-drug regimen versus four-drug regimen	89 91	7 (8%) 9 (10%)	Three-drug regimen is as effective as, and less toxic than the four-drug regimen	(26)

1.3.3 Risk factors for recurrent melioidosis.

Identified risk factors for recurrent melioidosis are summarized in table 1-5. An important limitation in the interpretation of these findings is that recurrence has been largely considered to represent relapse, and risk factors have not been considered separately for relapse versus re-infection. For example, it is unlikely that oral eradication treatment is associated with re-infection if this occurred after the completion of antibiotic therapy.

Table 1-5. Published risk factors for recurrent melioidosis.

Clinical severity	<p>Multifocal infection plus bacteraemia was associated with a 4.7 (95%CI, 1.6-14.1) times higher risk of recurrence compared with localized infection (29).</p> <p>Infection involving more than one non-contiguous organ with or without bacteraemia was associated with 1.8 higher risk of recurrence (95%CI, 1.0-3.4) (184).</p>
Parenteral treatment	<p>Initial parenteral treatment with ceftazidime had a 2-fold lower risk of recurrence (95%CI, 1.1-3.4) compared to treatment with other parenteral drugs (AMC or four-drug regimen) (29). However, this was not reproduced in later RCT studies (184).</p>
Oral treatment	<p>Poor drug compliance was found in 15 of 32 (47%) patients with recurrent melioidosis in Australia (64). A higher risk of recurrent melioidosis was reported in patients who did not take their drugs regularly (RR 4.9%; 95%CI, 1.2-20.3) (184).</p>
Oral treatment – duration	<p>In one study, treatment for more than 8 weeks was negatively associated with recurrent melioidosis (RR 0.4; 95%CI, 0.2-0.9) (29). In a second study, treatment for less than 12 weeks was associated with recurrent melioidosis (HR 5.7; 95%CI, 2.6-12.5) (26). The relationship between shorter duration of treatment and recurrent melioidosis was also supported by a review of patients in Thailand who were treated for 8 weeks versus 12-20 weeks, the rate of</p>

	recurrence decreasing significantly from 23% to 10% (59% reduction; 95%CI, 50-69%) (184).
Oral treatment regimen – AMC	Patients who received oral AMC compared to the four-drug regimen had 3.3 (95%CI, 1.4-9.0) times higher risk of recurrence (29). The inferior effectiveness of AMC was also shown in a later prospective RCT, in which the relative risk of recurrent melioidosis for the four-drug regimen was 0.4 (95%CI, 0.2-1.2) compared to that of AMC (184).
Oral treatment regimen – doxycycline monotherapy	Doxycycline monotherapy was associated with a higher rate of recurrent melioidosis compared to four-drug regimen (HR 12.0; 95%CI, 1.4-100.8) (27).
Oral treatment regimen – fluoroquinolone-based regimens	Ciprofloxacin or ofloxacin monotherapy was associated with a higher rate of recurrent melioidosis (30). In an RCT, patients treated with ciprofloxacin plus azithromycin had a higher recurrence rate compared to patients treated with three-drug regimen (22% vs 3%) (42).
Clinical manifestations	No association found with recurrence (29).
Underlying disease	No association found with recurrence (29;184).

1.3.4 Clinical manifestations of recurrent melioidosis.

Clinical manifestations of recurrent melioidosis are protean. Recurrent melioidosis may affect the same organs or different sites to those affected during the primary infection. Recurrent melioidosis was reported to affect the same organ as in the primary presentation in less than half (44%) of cases in Thailand (29). Of 27 patients with recurrence, 7 patients developed recurrence while still receiving oral eradication treatment. Seasonal variation of the onset of recurrence has not been reported.

Recurrent melioidosis can present as soon as 1 month after the start of oral eradication treatment (64). This may reflect clinical fluctuations in the course of recovery from the primary episode after switching from parenteral treatment to oral eradication treatment.

A patient reported by Mays and Ricketts initially presented with pneumonia in the right upper lobe and *B. pseudomallei* was isolated from cultures of sputum and bronchial lavage (157). He had recurrent melioidosis three weeks after antibiotics were discontinued with cultures positive from sputum. Puthuchearry *et al.* also reported a patient with the reappearance of a positive blood culture during oral eradication treatment with tetracycline (183). A further example is a case who had multifocal abscesses affecting the prostate, left elbow and right knees, who later had recurrent disease associated with a deep neck abscess and recurrent tibial osteomyelitis (131).

AMC does not reach high concentrations in the CSF, and a case report of recurrent melioidosis after AMC treatment following probable primary central nervous system infection has been reported (147). The primary presentation was *B. pseudomallei* bacteraemia and two generalized tonic-clonic convulsions. Her CT was reported to be normal. She was treated with a combination of TMP-SMX and doxycycline for 1 month during which she complained of nausea and vomiting. As a result, oral antimicrobials were switched to AMC. Two months later, she represented with a progressive left hemiparesis. A CT scan showed an epidural abscess, pus from

which grew *B. pseudomallei*. It is likely that there was a small infective lesion in the central nervous system during her primary infection which was not detected by the first CT scan.

1.3.5 Diagnosis of recurrent melioidosis.

Definite diagnosis of recurrence is based on the isolation of *B. pseudomallei* from a clinical specimen taken from a patient with a history of melioidosis. Cultures may be negative, however, from patients for whom there is a strong probability of recurrence. Furthermore, some patients may have a period of prolonged culture positivity during the primary infection which does not represent recurrence (64). Thus, careful clinical judgment is required in defining when and if recurrence has occurred.

1.3.6 Antimicrobial susceptibility of *B. pseudomallei* associated with recurrent melioidosis.

A cohort study conducted in northern Australia found that three of nine isolates (33%) associated with relapse cultured from patients treated with doxycycline monotherapy had acquired resistance to doxycycline (64). In the same study, one patient who relapsed after treatment with oral TMP-SMX had an organism that had acquired resistance to TMP-SMX. The development of resistance was found in 3 of 25 (12%) patients with recurrent melioidosis in Thailand (29). Development of drug resistance was associated with resistance in a patient who developed features of recurrence 10 days after completing a 10 week course of AMC treatment (221). Karcher *et al.* reported a patient with a second recurrent episode in which the isolate had become resistance to AMC after treatment with oral AMC for 5 months for the primary infection and a further 2 months for the first recurrence (131).

1.3.7 Antimicrobial treatment of recurrent melioidosis.

1.3.7.1 Parenteral antimicrobial treatment in acute phase.

Parenteral antibiotics used for primary melioidosis are effective drugs for the treatment of recurrence unless resistance has emerged to the drug used. Emergence of resistance to ceftazidime in patients with recurrence has not been reported, and this remains the treatment of choice (27;29;64). Specific guidelines for the duration of parenteral treatment for recurrent melioidosis have not been reported (27;29;64), and the standard guidelines of at least 10 to 14 days apply.

1.3.7.2 Oral antimicrobial treatment in eradication phase.

Successful treatment of recurrence has been reported for TMP-SMX with or without doxycycline or AMC (27;29;64). In practice, AMC may be used to treat recurrence if the primary episode was treated with TMP-SMX (personal communication, Chaowagul, W.). However, the effectiveness of AMC as an oral eradication treatment regimen is lower than a TMP-SMX based regimen, and the latter should remain the treatment of choice for recurrence due to relapse and re-infection.

Duration of oral treatment required for recurrent melioidosis is uncertain (27;29;64). Durations of longer than 20 weeks may be required (27), but as for primary infection this should be guided by clinical response.

1.3.8 Relapse or re-infection as a cause of recurrent melioidosis.

Previous studies have largely treated recurrence as a homogenous condition, with cases being assumed to have resulted from relapse of a persistent focus of infection. However, recurrence can be caused by relapse following failure to eradicate bacteria responsible for the primary infection, or by re-infection after an exposure to a new bacterial strain. Distinguishing between the two is important since clinical trials of

oral therapy for melioidosis commonly use recurrent disease as a marker for treatment failure. Several small typing studies involving 5 to 25 patients with recurrence that compared bacterial isolates from the first and second episode of infection have suggested that recurrent melioidosis is not always due to relapse.

The first study performed to distinguish between the relapse and re-infection was reported from Thailand in 1993 (80). This study included 25 patients with recurrent melioidosis presenting between 1986 and 1991 (29). *Bam*HI restriction digest ribotyping was used to compare strains from the first and second presentation (80). Primary and recurrent isolates were identical or highly similar for 23 patients, while 2 recurrent episodes (8%) were caused by isolates that were genetically different from the isolate causing primary infection, indicating that relapse was the dominant cause of recurrence rather than re-infection.

A study in northern Australia published in 1995 compared *B. pseudomallei* isolated from 10 patients with primary and recurrent melioidosis using RAPD (105). These strains were later re-examined using PFGE (64). The mean time period between primary episode and recurrence was 8 months. Two of 32 recurrent episodes (6%) were classified as re-infection; the interval between primary infection and recurrence was 1.2 and 4.8 years, respectively (64;105). One patient with chronic osteomyelitis was thought to have had relapse with 1 of 2 strains originally infecting the patient (64).

A study from Malaysia of 5 patients with recurrence examined strains using *Bam*HI ribotyping and pulsed-field gel electrophoresis (PFGE) of *Xba*I digests (240). One of five patients (20%) with recurrent episodes was infected with a strain that was clearly distinct from the original primary isolate. These studies are consistent with the idea that not all patients with recurrent disease have relapse. However, they involved small numbers of patients, and the typing methods used are not unambiguous in that PFGE banding patterns could potentially change in the host over time.

1.3.9 Polyclonal infection could cause misclassification of recurrence.

An important possible source of error when determining the cause of recurrence could arise if simultaneous infection with more than one strain of *B. pseudomallei* was common and different strains from the primary and recurrent episode were picked by chance for genotyping; this would lead to misclassification of relapse as re-infection. In a study of recurrent melioidosis, a sweep of colonies was taken from the isolation plates in an attempt to avoid this, but only single colonies were subsequently used for typing; therefore, selection of a single strain is likely to have occurred (80).

Infection with multiple strains of *B. pseudomallei* was first demonstrated by Sexton *et al.* (198). In this study, 74 clinical isolates of *B. pseudomallei* from 34 patients were examined. Ten ribotypes were identified from a combination of restriction fragment polymorphisms from *EcoRI*, *SalI*, *HindIII*, and *PstI* chromosomal digests. Isolates with two different ribotype patterns were found in one patient (3%), one each in sputum and urine. This prospective study demonstrated that infection with more than one strain can occur. A second study reported that a patient with two recurrent episodes was initially infected with two strains (80). A recent retrospective study suggested that polyclonal infection may be relatively common. An evaluation of samples from 18 patients presenting to Sappasithiprasong Hospital, northeast Thailand between 1992 and 1993 was conducted (176). Between 10 and 40 colonies were selected from primary culture plates which were compared using a combination of *BamHI* ribotype pattern and PFGE pattern of *XbaI* DNA digests. Five of 18 patients (28%; 95%CI, 10%-53%) were shown to be infected with more than one genotypic strain. Furthermore, patients with polyclonal infection had more severe disease and a poorer outcome. This study suggested that the incidence of polyclonal infection may be higher than previously thought. However, this study was small and conducted in a selected population, which could introduce bias.

1.3.10 *B. pseudomallei* genotypes in the environment could influence the classification of recurrence.

Re-infection could be misclassified as relapse in the event that the population genetic structure of *B. pseudomallei* in the environment was highly clonal. In this case, an individual could have independent infections caused by the same bacterial clone. An understanding of the bacterial population genetic structure in the environment is required in order to assess this possibility.

In 1995, Hasse *et al.* used RAPD to determine the genotypes of *B. pseudomallei* strains associated with an outbreak of melioidosis in goats on a farm near Darwin, Australia and from serial soil samples from the paddock between 1992 and 1993 (106). Fourteen of the 15 goats were infected with the same strain. Only 3 of 24 soil isolates were genetically identical to this outbreak strain. One goat was infected by a different strain, and this was identified from 9 of the soil samples. The remaining 12 soil isolates gave two further RAPD patterns that were not identified from goat strains. This study confirmed that soil isolates can be identical to epidemiologically related clinical isolates, and showed that there was a degree of genetic diversity of *B. pseudomallei* over a restricted area.

In 1997, Vadivelu *et al.* used *Bam*HI ribotyping and PFGE of *Xba*I digests to examine 49 isolates of *B. pseudomallei* from 49 sporadic melioidosis cases in Malaysia over an 18-year period (242). Five ribotype patterns were identified; in addition, PFGE revealed different strains within the same ribotype. Two pairs of isolates from unrelated cases gave an indistinguishable PFGE pattern. The first pair was from two patients separated by 7 years, and the second pair was from patients living in different towns. This study suggested that independent infections can occur with an indistinguishable strain.

In 2004, Cheng *et al.* used a combination of PFGE and MLST to examine 87 *B. pseudomallei* isolates from clinical cases and the environment (36). A total of 48 STs were defined, suggesting genetic diversity of the population. Using MLST, Vesaratchavest *et al.* examined 183 clinical and 83 soil isolates (245). A total of 123 STs were defined. These studies suggest that *B. pseudomallei* in the environment and associated with disease is genetically diverse. One issue that is not addressed by these two studies is whether specific STs predominate over a small area of land, such as that farmed by a single farmer. If this were so, then repeated exposure could lead to re-infection with a dominant clone.

1.4 Aims of this dissertation.

1.4.1 To undertake a large study in northeast Thailand to determine the proportion of recurrent melioidosis that is due to relapse versus re-infection as defined by bacterial genotyping of primary and recurrent strains using a combination of PFGE and MLST. This will include the following nested studies:

- i. Relapse could be misclassified as re-infection in the event that polyclonal infection during the primary episode was common, and different strains were picked from the primary and recurrent cultures for genotyping. The rate of primary infection with more than one strain will be addressed during a large prospective study in northeast Thailand in which genotyping will be applied to multiple colonies from multiple sites from a given patient.
- ii. Re-infection could be misclassified as relapse in the event that the population genetic structure of *B. pseudomallei* in the environment was highly clonal. This will be studied by defining the genotypes of *B. pseudomallei* isolated from soil sampled from a small defined area of land in northeast Thailand, together with an analysis of MLST data held in the public database.

1.4.2 In the event that recurrence is not simply due to relapse, a database of nearly 1,000 patients with melioidosis presenting to Sappasithiprasong Hospital will be used to:

- i. define the specific risk factors for relapse and for re-infection
- ii. compare and contrast the clinical features of relapse and re-infection
- iii. develop a clinical scoring system that can be used to predict whether recurrent melioidosis is due to relapse or re-infection

Chapter 2. Materials and Methods.

2.1 Chemical and reagents.

All chemicals and reagents were obtained from Sigma-Aldrich Company Ltd. or Merck Ltd. Bacterial culture media were obtained from Oxoid. Sterile plastic ware was obtained from Falcon, Becton Dickinson Ltd. and Terumo. The ingredients and amounts used to prepare reagents were described in appendix.

2.2 Bacterial culture and storage conditions.

2.2.1 Liquid medium.

B. pseudomallei was grown in TSB under static conditions at 37°C in air. Cultures were routinely grown in 3 ml of media contained in 15 ml universal containers.

2.2.2 Solid media.

All agar plates used in this study were prepared as recommended by the manufacturer. *B. pseudomallei* was routinely cultured on Ashdown's Selective Agar at 37°C in air. Other media used during clinical studies are specified in the relevant results chapters.

2.2.3 Bacterial storage.

B. pseudomallei was suspended in TSB containing glycerol (15%, v/v) and stored in locked freezers at -80°C.

2.3 Laboratory facilities.

All experimental work involving viable bacterial cells was performed in a Category 3 containment facility under negative pressure of –50 Pascals. All work that may create aerosols or splatter was carried out inside a class II biological safety cabinet.

2.4 *B. pseudomallei* identification.

All isolates were identified in the clinical diagnostic laboratory in Ubon Ratchathani as *B. pseudomallei* based on growth on Ashdown's agar with typical colony morphology after 3 or 4 days of incubation at 37°C in air, a positive oxidase test, and resistance to Gentamicin (disc strength 10µg) and Colistin (disc strength 10µg) after overnight incubation at 37°C in air on Columbia agar (250). Further confirmation was obtained using a highly specific latex agglutination reaction (262). In brief, a small amount of colony is emulsified using a sterile toothpick into a solution containing latex particles coated with a monoclonal antibody specific for a 200-kD surface antigen of *B. pseudomallei*. The reaction is read within 1-2 min. The presence of agglutination is taken as a positive reaction. *E. coli* is used as a negative control. API 20NE biochemical strips were used as recommended by the manufacturer (Bio-Merieux, Inc.), if any uncertainty remained about the bacterial species. Typical API profiles are 1156577 or 1156576 by APILAB Plus software; this gives an automated interpretation on a computer workstation (bioMerieux, 2005). To exclude the possibility that some isolates were *B. thailandensis*, all isolates were tested for their inability to assimilate arabinose. This was performed in batches at the end of the year in the Wellcome Unit, Bangkok. *B. pseudomallei* is negative and *B. thailandensis* is positive (262). Isolates were point inoculated onto agar plates containing arabinose as

the only source of nutrition. *B. pseudomallei* fails to grow on this medium after 48 hours incubation at 37°C in air, while *B. thailandensis* shows growth.

2.5 PFGE.

2.5.1 Introduction.

PFGE enables DNA megabase-sized fragments to be separated according to size, allowing chromosomal RFLPs (generated by rare cutting enzymes) to be examined. PFGE has been used both for epidemiological studies and to examine the physical map of bacterial chromosomes. A specialized electrophoresis chamber is used (the Contour-clamped Homogenous Electrophoresis Field (CHEF) DRIII was used in the current study), in which an agarose gel is positioned between 3 sets of electrodes forming a hexagonal shape around the gel. Current is applied to one set of electrodes creating an electric field across the tank. The current is then switched to a second set of electrodes, changing the direction of the field. After a defined period of time it switches to the third set of electrodes, shifting the field again. This pulsed electric field causes the DNA to periodically re-orientate its direction of migration as it moves up the gel (60;230). The rate of migration through the gel is determined by the size of each DNA fragment, as smaller DNA fragments are able to re-orientate faster than large ones (99).

Many diverse organisms have been examined using a variety of different restriction enzymes. The choice of restriction enzyme will depend on the average number of specific restriction sites in a particular bacterial genome, and the required average number of the resulting fragments. *SpeI* is the enzyme usually used to cut *B. pseudomallei*. This cuts between 5'-A/CTAGT-3' sites, and cuts the *B. pseudomallei* genome into approximately 20 bands ranging in size from 20 to 800 kb (34).

2.5.2 Procedure of PFGE.

2.5.2.1 Preparation of DNA.

To prepare chromosomal DNA, bacterial cells were streaked onto an Ashdown's agar plate and incubated at 37°C overnight. Bacterial colonies were then harvested and suspended in a tube containing 2 ml of SE buffer (75 mM NaCl and 25 mM EDTA, pH 7.5). The solution was mixed to obtain a homogenous solution and the concentration of *B. pseudomallei* was adjusted to an optical density at 600 nm of 1.2. The solution was then mixed with an equal volume of molten 2% low melting agarose (Invitrogen) and pipetted into PFGE plug moulds (Bio-Rad Laboratories, Richmond, CA). Agarose blocks were maintained at 56°C for 24 hours in lysis buffer (0.1% SDS and 25 mM EDTA, pH 8.0) containing 500 µg/ml proteinase K (Invitrogen). The cell debris and proteinase K were then removed by washing three times with TE buffer (10 mM Tris and 10 mM EDTA). DNA plugs were then equilibrated in TE buffer for 1 hour at room temperature.

2.5.2.2 Restriction endonuclease digestion and PFGE.

Before restriction enzyme digestion, agarose plugs were divided into 2 mm thick blocks and equilibrated for 30 minutes in 100 µl of 1x restriction enzyme buffer. The buffer was aspirated and samples were then digested with a restriction enzyme in 100 µl of final solution containing 10 units of *SpeI* enzyme (5'-ACTAGT-3'; New England Biolabs, Beverly, MA). The blocks were incubated at 37°C overnight.

PFGE was performed using the CHEF-DRIII system (Bio-Rad Laboratories, Richmond, CA) in gels of 1% gel agarose in 0.5x TBE buffer (45 mM Tris, 45 mM boric acid and 1 mM EDTA) for 44 hours at a temperature of 14°C, field strength of 6 V/cm using the following parameters: initial switch time, final switch time, and runtime for block I, 10 to 60 sec for 18 hours; block II, 30 to 40 sec for 18 hours; and block III,

50 to 90 sec for 8 hours. Bacteriophage lambda concatemers starting at 48.5 kbp and increasing to approximately 1,000 kbp were run as the standard (Promega). Gels were stained with 2.0 µg/ml ethidium bromide (EtBr) for 30 minutes, washed in 250 ml distilled water for 4 hours, and photographed under UV light using the Gel Doc 2000 system (Bio-Rad Laboratories, Richmond, CA). Analysis of the PFGE banding patterns was performed using BioNumerics (version 2.5) software (Applied Maths, Belgium).

2.5.3 Interpretation of PFGE result.

PFGE was performed as the initial screen to determine whether two isolates were genetically the same or different. This was applied to clinical and environmental *B. pseudomallei* isolates. Two strains with identical banding patterns were considered to belong to the same clone and no further genotyping was undertaken. Strain pairs in which one or more bands differed were evaluated further using an approach based on MLST.

2.6 MLST.

2.6.1 Introduction.

MLST schemes and databases have been described for a number of important bacterial pathogens including *Neisseria meningitidis* (152), *Streptococcus pneumoniae* (85) and *Staphylococcus aureus* (84). An MLST scheme for *B. pseudomallei* was described by Godoy *et al.* in 2003 (98). MLST identifies variation within fragments of seven housekeeping genes that would be predicted to be selectively neutral (152). Each *B. pseudomallei* isolate is defined by a string of seven integers (the allelic profile), which correspond to the allele numbers at the seven loci, in the order *ace-gltB-gmhD-lepA-lipA-narK-ndh*. Each unique allelic profile is considered a clone and is assigned a ST, which also provides a convenient descriptor for the clone (152;211). An MLST

database containing the sequences of all alleles, the allelic profiles, and information about the *Burkholderia* isolates, together with analysis tools, is maintained at Imperial College (London, United Kingdom) and can be found on the *Burkholderia* pages of the MLST website (www.mlst.net).

2.6.2 Procedure of MLST of *B. pseudomallei*.

2.6.2.1 Preparation of DNA.

To prepare chromosomal DNA, bacterial cells were streaked onto Ashdown's agar and incubated at 37°C overnight. A single colony was inoculated into TSB and incubated in air at 37°C overnight. Dense bacterial suspensions were boiled for 15 minutes. An aliquot of each boiled suspension was checked for the absence of living bacteria, and then DNA was extracted from the suspensions using the Wizard Genomic DNA Purification kit (Promega).

2.6.2.2 Primers.

The primer pairs used to amplify housekeeping gene fragments on chromosome 1 are shown in table 2-1.

2.6.2.3 PCR.

PCR was performed using a PTC-0200 DNA engine (MJ Research, Cambridge, USA). Reactions were carried out in a total volume of 50 µl. Each reaction contained 1X reaction buffer, 1.5 mM MgCl₂, 0.7 µM of each primer, 5 µl of working template DNA, 200 µM of deoxynucleotide triphosphate (dNTP) and 2.5 U of *Taq* DNA polymerase (Promega, USA). Cycling conditions were an initial denaturation at 95°C for 4 min followed by 34 cycles of denaturation at 95°C for 30 s, the annealing temperature shown in table 2-2 for 30 s, and extension at 72°C for 60 s. The samples

were then maintained at 72°C for 10 min and cooled to 4°C. The amplification product was visualized using 2% agarose gel electrophoresis followed by staining with ethidium bromide and visualization using a Gel Doc 2000 system (Bio-Rad Laboratories, Richmond, CA).

Table 2-1. Primer pairs and PCR cycling conditions used for MLST of *B. pseudomallei*.

Primer name	Gene function	Primer Sequence (5'-3')	Annealing Temp. (°C)	Product size (bp)
Outer_aceFwd	Acetyl coenzyme A reductase	GCCGCTCGGCGCTTCTCAA	60	767
Outer_aceRev		AGCCGCCGTTACAGCGAAAAATC		
Outer_lepAFwd	GTP-binding elongation factor	GCATCACGACGCCGACGTAGTTGT	60	724
Outer_lepARev		CTGGCGGGCCTTTTCGGCTAAA		
Outer_lipAFwd	Lipoic acid synthetase	ACGATCCGACCGCCCAAGCAGAAGG	60	807
Outer_lipARev		ACGTACTCGCGCACCGGCAGATGG		
Outer_narKFwd	Nitrite extrusion protein	GCCGTCAGCGTGAGCCTCGTCT	60	767
Outer_narKRev		AGCCCGCGTTCTTGCAACCACA		
Outer_ndhFwd	NADH dehydrogenase	TATCCCGCCGATCAGAAACAGTCC	60	719
Outer_ndhRev		GCCCGGCTCGCCCTCGTC		
Outer_gltBFwd	Glutamate synthase	TGTCGGGGCCCGTCTTTCATCT	63	727
Outer_gltBRev		ATCAGCACCGAGGCGGCATACGAC		
Outer_gmhDFwd	ADP glycerol-mannoheptose epimerase	TCGCGCAGGGCACGCAGTT	65	705
Outer_gmhDRev		GGCTGCCGACCGTGAGACC		

2.6.2.4 Clean-up of PCR products.

The amplified DNA fragments (25 µl) were precipitated by mixing with 60 µl of 20% polyethylene glycol 8000/2.5M NaCl for 1 h at room temperature or 4°C overnight. Following centrifugation at 13,000 rpm for 40 min, the supernatant was discarded and the pellet was washed with 600 µl of 70% ethanol. The tube was centrifuged again as above. The supernatant was then removed and the DNA pellet was dried at 37°C for 1 h and resuspended with 10 µl of sterile distilled water.

2.6.2.5 Sequencing.

The DNA fragments on each strand were sequenced using a DYEnamic ET Dye Terminator Kit (Amersham) and a MegaBACE 500 sequencer with nested primer pairs shown in table 2-2.

Table 2-2. Primer pairs used for sequencing of amplification products.

Primer name	Primer Sequence (5'-3')
Nested_aceF	GCTCGGCGCTTCTCAAAA
Nested_aceR	ATGTCCGTGCCGATGTAGC
Nested_lepAF	TTTCGCTTGATCGGCACTG
Nested_lepAR	CGAACCACGAATCGATGATGA
Nested_lipAF	CATACGGTGTGCGAGGAAGC
Nested_lipAR	GCAGGATCTCGTCGGTCGTCT
Nested_narKF	GCCACCCGCTCCGCGTGAGC
Nested_narKR	AGCCCGCGTTCTGCAACCACA
Nested_ndhF	GCAGTTCGTCGCGGACTATCTC
Nested_ndhR	GGCGCGGCATGAAGCTC
Nested_gltBF	GGCGGCAAGTCGAACACGG
Nested_gltBR	GCAGGCGGTTTCAGCACGAG
Nested_gmhDF	TCGCGCAGGGCACGCAGTT
Nested_gmhDR	GTCAGGAACGGCGCGTCGTAG

2.6.3 Interpretation of sequence data.

SeqmanTMII software version 6.1 (DNASTAR Inc., USA) was used to analyse nucleotide sequences in both forward and reverse strands of the DNA sequence at each housekeeping gene. Sequence trace of both strands were aligned to each other to obtain a consensus sequence prior to assignment of allelic number. The allelic numbers of previously described loci were assigned using the *B. pseudomallei* MLST website (<http://www.mlst.net/>). Sequence that was not in the database was checked by resequencing, assigned as a new allele, and deposited in the MLST allele database. Following the standard MLST protocol, each allele was assigned a different allele number to create an allelic profile (string of seven integers) in the order *ace-gltB-gmhD-lepA-lipA-narK-ndh*. Each unique allelic profile was considered a clone and was used to define the ST.

Chapter 3. Recurrent infection in melioidosis patients in northeast Thailand is frequently due to re-infection rather than relapse.

3.1 Chapter content.

Recurrent melioidosis is widely ascribed to relapse caused by a bacterial strain that has persisted within a sequestered focus, rather than to re-infection with a different strain. This is based on the findings of previous small studies in which isolates from the first and second episodes of infection in a given patient usually had the same genotype (64;80;105).

The aim of the study was to perform a large, definitive prospective evaluation of patients with recurrent melioidosis presenting to Sappasithiprasong Hospital in northeast Thailand. This was achieved by:

1. PFGE on all primary and recurrent isolates from 116 patients with recurrent melioidosis.
2. Comparing PFGE banding patterns of isolates from primary and recurrent infection for each patient, and undertaking a sequence-based approach based on MLST for strains with different patterns.
3. Investigating the difference in time to presentation of relapse and re-infection, and estimating the risk of re-infection for patients who have had a primary episode of melioidosis.

3.2 Materials and Methods.

3.2.1 Patients and bacterial isolates

Patients were prospectively recruited by a study team based at Sappasithiprasong Hospital, Ubon Ratchathani, northeast Thailand. Study patients were adults (≥ 15 years) with culture-confirmed melioidosis who presented between June 1986 and July 2004. All patients were followed up until July 2005. Patients with suspected melioidosis were actively sought during twice-daily rounds of the medical and intensive care wards, together with passive surveillance of surgical and pediatric wards via the hospital diagnostic microbiology laboratory. Detailed clinical information was recorded, including a past history of culture-proven melioidosis. Blood and throat swab samples were collected from all patients for culture. Other specimens (urine, pus, and surface swabs of skin lesions) were collected where available. Microbiology specimens were cultured for the presence of *B. pseudomallei* by standard methods (250). Culture-confirmed cases were followed daily until discharge or death. Details of history, examination, laboratory results, antimicrobial treatment and clinical course were maintained on a password protected computer database. Isolates were stored in TSB with 15% glycerol at -80°C . Ethical approval was obtained from the Ministry of Public Health, Royal Government of Thailand.

3.2.2 Genotyping.

For all patients with more than one episode of disease, the first *B. pseudomallei* isolate cultured on the first and subsequent episodes of melioidosis were examined by PFGE. Isolates from primary and recurrent disease for a given patient that were different by one or more bands were further examined by MLST. PFGE and MLST were performed as described in chapter 2.

3.2.3 Colony morphology.

Colony morphology was characterised using a published morphotyping algorithm (24). Isolates were spread plated to achieve single colonies on Ashdown's agar and incubated for 4 days at 37°C in air. Colony morphology type was compared between the first and subsequent cultures for each patient.

3.2.4 Definitions.

Recurrent melioidosis was defined as clinical features of infection in association with one or more cultures positive for *B. pseudomallei* in a patient with a history of one or more previous episodes. This included patients who had completed treatment for the previous episode, together with patients who had recurrent symptoms while receiving oral antibiotics for melioidosis following an initial clinical and microbiological response to antibiotic therapy. Time to recurrent disease was measured from the start of oral antimicrobial treatment, irrespective of whether the patients received intravenous therapy.

Disease relapse and re-infection were defined on the basis of bacterial genotyping of isolates from the first and subsequent episode(s). Isolates from the same patient with identical banding patterns on PFGE were considered to represent a single isolate, and these patients were classified as having relapse. Isolates from the same patient that differed by one or more bands were examined further using a screening approach based on MLST. This used the assumption that a difference in just one of seven MLST loci is sufficient to assign different STs. Two of the seven MLST loci, *narK* and *gmhD*, are highly variable within the bacterial population. The sequence of *narK* was determined first, followed by sequencing of *gmhD* for isolate pairs with the same allele *narK*. Isolates from the same patient that differed at one or both of these

loci were considered to represent re-infection. Those with sequence identity at both *narK* and *gmhD* were further characterized using the remaining five MLST loci to define the full ST.

3.2.5 Analysis.

Statistical tests were performed using Stata 9.0 (College Station, Texas, United States). Proportions were compared by Chi-square and Fisher's exact test, as appropriate. Continuous variables were compared by the Wilcoxon rank-sum test.

3.3 Results.

A total of 147 patients presented with 167 episodes of culture-proven recurrent melioidosis between June 1986 and July 2004. Of these, five patients had three episodes of recurrence, ten patients had two episodes of recurrence and the remainder had a single recurrence. Paired isolates were not available for 32 patients. After these patients were excluded, isolates from 115 patients with a total of 122 episodes of recurrent disease were available for typing. The time to recurrence for 122 episodes ranged from 1 to 454 weeks (8.7 years). The median time to recurrence was 39 weeks, with an interquartile range (IQR) of 12 to 115 weeks.

3.3.1 The proportion of recurrent disease attributable to relapse and re-infection.

PFGE banding patterns were identical between the first and subsequent infective isolates for 90 of 122 episodes, including those episodes for five of the six patients with more than one recurrence. PFGE banding patterns differed between isolate pairs in 32 episodes. This included an episode in one patient with two recurrent infections. The strain from the first recurrence had the same pattern as that of the strain from the primary infection, but the second recurrence was caused by an isolate with a different banding pattern. The number of bands different between pairs ranged from 1 to 15 (median, 6 bands; IQR 4 to 7 bands). Sequence analysis of one or both of *narK* and *gmhD* indicated that isolates were different for 30 of the 32 episodes. Two isolate pairs that were one and four bands different, respectively, on PFGE were identical by full sequence typing of all seven loci. Thus, 92 episodes (75%) of recurrence represented relapse and 30 episodes (25%) were due to re-infection with a new strain.

There were five isolate pairs that were classified as closely related by PFGE (up to three band differences) by the criteria of Tenover *et al.* (230), of which four (80%)

were found to be different by sequenced-based typing. Of the 14 pairs classified as possibly related (four to six band differences), 13 (93%) were found to be different by sequence-based typing. All 13 pairs with greater than six band differences were different based on sequence-based typing.

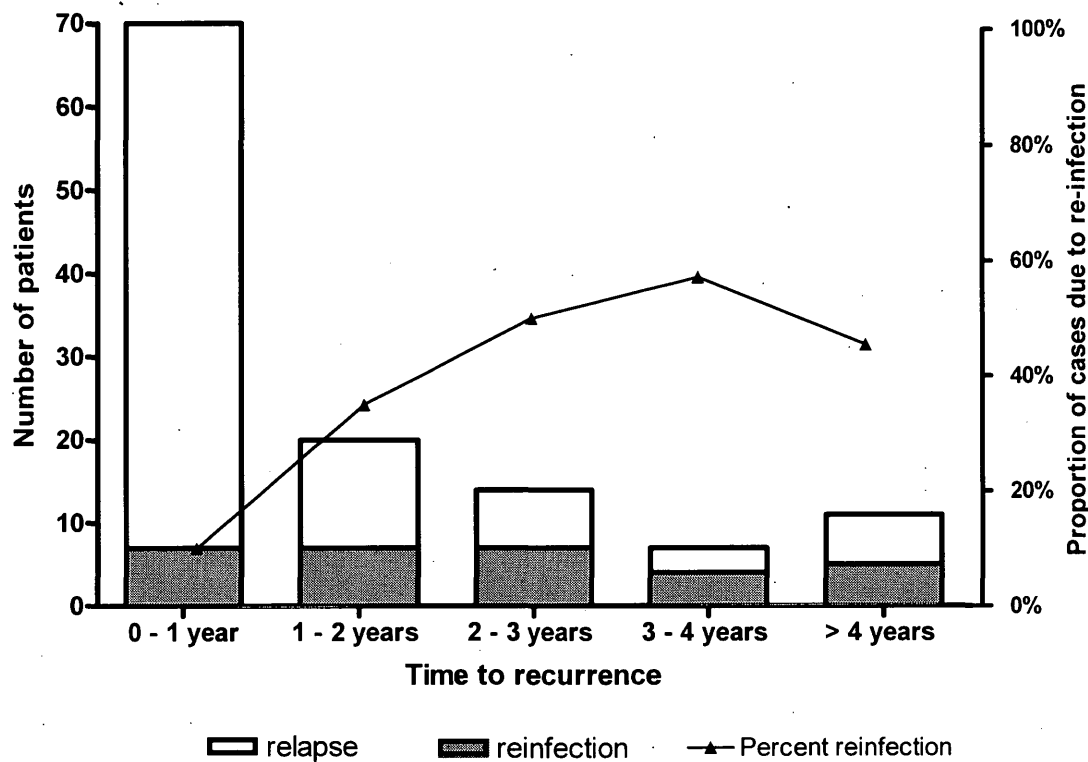
3.3.2 Time to relapse and re-infection.

Time to recurrence differed for episodes due to relapse compared with those due to re-infection. Those caused by relapse had a median interval of 26 weeks (IQR 10-72 weeks; range 1-454 weeks), while those due to re-infection had a median interval of 111 weeks (IQR 59-164 weeks; range 1-363 weeks) ($P<0.001$). Time to recurrence and the proportion due to relapse and re-infection are illustrated in figure 3-1. A total of 70 episodes (57%) occurred within 12 months of the primary infection. Recurrent disease was due to relapse in 63 of 70 episodes (90%) that occurred within the first year of the primary episode, in 13 of the 20 episodes (65%) in the second year after the primary episode, and in 16 of 32 episodes (50%) more than 2 years after the primary episode.

3.3.3 Colony morphotype between primary and recurrent strains.

A difference in colony morphotype between primary and recurrent strains was uncommon. There were only 12 instances where the colony morphology type was different between the isolate pairs associated with primary and recurrent infection. Of these, five (42%) represented relapse.

Figure 3-1. Time to recurrent disease and proportion of cases due to relapse and re-infection.



3.4 Discussion.

In an earlier study performed in our population, isolate pairs from 23 of 25 patients with recurrent disease had identical or highly similar ribotype patterns, while those for two patients were different (80). Follow up was performed for 5 years, and 19 episodes occurred within the first 12 months. A further study performed in Thailand reported different PFGE banding patterns for isolate pairs from 4 of 35 patients with recurrent melioidosis (138). In Australia, where recurrent melioidosis was observed in 27 (13%) patients over a follow-up period of 10 years, the majority of cases (25 of 27) were due to relapse as defined by RAPD analysis or PFGE. In these patients, relapse occurred between 1 and 27 months (mean 8 months) after the primary presentation, while the two re-infections occurred after 14 months and 4.8 years, respectively (64). In a Malaysian study, four of five recurrences were due to relapse of the original infecting strain, as based on ribotyping (240). Our contrasting findings are likely to reflect prolonged patient follow-up, with the longest interval between primary infection and recurrence being more than 8 years. Use of different typing methods may also be important, since ribotyping may be less discriminatory than PFGE (175;242).

The criteria of Tenover *et al.* (230) have been widely adopted as the standard for the interpretation of DNA macrorestriction banding patterns in microepidemiological investigations. DNA macrorestriction patterns that differ by up to three bands are interpreted as being closely related, and differences of four to six bands are interpreted as possibly related. Use of these criteria in this study without the use of further genotyping would have led to the misclassification of four patients with re-infection into the relapse group and one patient with relapse into the re-infection group.

The predictive power of using colony morphology type as a surrogate for strain typing in patients with recurrence was examined here and not found to be useful. The presence of different colony morphotypes for isolates associated with primary and

recurrent infection did not predict re-infection. A study by Chantratita *et al.* (24).

described seven *B. pseudomallei* colony morphotypes from clinical and environmental specimens. However, 93% of isolates from clinical specimens were type I.

In this study, recurrent disease within 12 months was mostly due to relapse of the original infecting strain, a finding that is consistent with previous results. However, the proportion of recurrent disease due to re-infection increased after this time and after 2 years, recurrence was related in equal proportions to re-infection and relapse. The number of re-infections remained similar for each year of the study. The estimated rate of re-infection in people who have had an episode of melioidosis is 5 to 8 per 1,000 patients per year, which far exceeds the annual incidence in the community for primary melioidosis of 4.4 per 100,000 population (222). This illustrates a very high susceptibility for re-infection with *B. pseudomallei*.

This study has important implications for future studies. After intervals exceeding 12 months, recurrent disease often represents re-infection rather than recrudescence of the original infecting isolate. Studies of oral antibiotic eradication therapy for melioidosis that use recurrent disease as a marker of drug efficacy need to undertake bacterial typing to accurately distinguish relapse from re-infection, analogous to studies of antimalarials. Future typing studies should consider the use of sequence-based verification of the results for isolates that are different on PFGE. Patients with previous melioidosis represent a group of individuals who are susceptible to subsequent infection. Re-infection following re-exposure to environmental *B. pseudomallei* is more common than had previously been appreciated.

3.5 Chapter summary.

This study used a robust combination of genotyping techniques to define the proportions of recurrent melioidosis due to relapse versus re-infection. Re-infection was shown to be a more common cause of recurrent melioidosis than previously thought and occurred at the same incidence every year, while relapse was more likely to occur within the first year after the primary episode. These findings have important implications for the prevention of re-infection in those patients with a history of primary melioidosis.

Chapter 4. Simultaneous infection with more than one strain of *B. pseudomallei* is uncommon in human melioidosis.

4.1 Chapter content.

Recurrent melioidosis is ascribed to re-infection when the genotype of the bacterial isolates cultured at the first and subsequent disease episodes are not the same. One possible criticism of this approach is that 're-infection' could actually represent relapse in the event that the primary infection was caused by simultaneous infection with multiple *B. pseudomallei* strains, followed by chance selection of different strains from the two episodes for genotyping. The chance of mistake occurring is based on the rate of polyclonal *B. pseudomallei* infection. In a previous study of 18 patients presenting to a hospital in northeast Thailand between 1992 and 1993 in which 10 to 40 colonies were picked from primary culture plates, five cases (28%) were shown to have infection with more than one genotypic strain using a combination of ribotyping and PFGE (175). Of these, four patients had isolates with two genotypic patterns and one patient had three. Furthermore, patients with polyclonal infection had more severe disease and a poorer outcome.

The aim of this study was to define the rate of polyclonal infection in a large group of unselected patients presenting to a hospital in northeast Thailand with their first episode of infection. This was achieved as follows:

1. A large, prospective study was conducted to collect appropriate samples from patients with suspected melioidosis.

2. Multiple colonies from multiple samples from a given patient were compared using a combination of PFGE and MLST.
3. An analysis was performed to:
 - i. determine the rate of polyclonal infection, and to
 - ii. compare the mortality associated with polyclonal versus single clone infection.

4.2 Materials and Methods.

4.2.1 Patients and specimen collection.

Patients were prospectively recruited by a study team based at Sappasithiprasong Hospital, Ubon Ratchathani, northeast Thailand, between 28th June and 29th September 2006. The study was approved by the Human Research Ethics Committee of the Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand. Patients with suspected melioidosis were actively sought during twice-daily rounds of the medical and intensive care wards, together with passive surveillance of surgical and pediatric wards. Detailed clinical information was recorded, including a documented history of culture-proven melioidosis. Multiple samples were taken from suspected cases within 72 hours of admission. A 15ml blood sample was taken and divided between a BacT/ALERT® FA bottle (BioMérieux, North Carolina) for standard culture (5ml), and an Isolator10 lysis centrifugation tube (Oxoid, Basingstoke, Hampshire, UK) (10ml) to allow identification of primary colonies. Samples were taken for culture of sputum/tracheal aspirate, throat swab, urine, pus or surface swab from wounds and skin lesions, as appropriate or available. Specimens were hand carried to the on-site research laboratory at the end of each ward round.

The study was powered to compare the mortality associated with polyclonal versus single clone infection. Assuming a polyclonal infection rate of 30% (based on the study reported by Pitt *et al.* (176) which was undertaken in the same population), and an overall mortality rate in our melioidosis population of ~45%, at least 102 patients are required to detect a difference in mortality rate of 70% (polyclonal infection) and 35% (clonal infection), respectively with 80% power. The number of colonies required from each sample was determined; at least ten colonies are needed to detect the presence of two strains in a ratio of 1:9 with 85% power. All analyses were calculated using Stata 9.0 (College Station, Texas, United States).

The sample size calculations have been reproduced below:

```
. sampsi 0.35 0.70, r(0.3) p(0.80)
```

Estimated sample size for two-sample comparison of proportions

Test Ho: $p_1 = p_2$, where p_1 is the proportion in population 1

and p_2 is the proportion in population 2

Assumptions:

alpha = 0.0500 (two-sided)

power = 0.8000

p1 = 0.3500

p2 = 0.7000

n2/n1 = 0.30

Estimated required sample sizes:

n1 = 78

* n2 = 24

```
. sampsi 0 0.1, onesample p(0.85)
```

Estimated sample size for one-sample comparison of proportion

to hypothesized value

Test Ho: $p = 0.0000$, where p is the proportion in the population

Assumptions:

alpha = 0.0500 (two-sided)

power = 0.8500

alternative p = 0.1000

Estimated required sample size:

n = 10

4.2.2 Bacterial isolates.

The objective of culture was to achieve single *B. pseudomallei* colonies on primary plates inoculated with material taken directly from the clinical sample. The rationale for this was to obtain a true picture of the bacterial population in the primary specimen, and to avoid the use of sub-culture in enrichment broth during which one strain of *B. pseudomallei* could outgrow a second.

The 10ml blood sample was collected into a pre-evacuated Isolator10 tube lysis centrifugation blood culture tube (Wampole, UK). The isolator10 tubes contain SPS, saponin and polypropylene glycol in aqueous solution. Isolator tubes were inverted at least 10 times after inoculation to ensure adequate mixing, and then transported immediately to the on-site microbiology laboratory and centrifuged at 1500 rpm for 30 minutes. The supernatant (9 ml) was aseptically removed to leave 1 ml of fluid plus sediment. The sediment was homogenized using a sterile pipette, and 0.5 ml was spread plated onto each of two plates: horse blood and Ashdown's selective agar.

Urine was collected into a sterile container and transported immediately to the laboratory. Using a sterile calibrated loop, 1 µl of fresh, unprocessed urine was streaked onto one half each of a MacConkey agar plate and an Ashdown's agar plate which were incubated at 37°C for 2 days or 4 days, respectively. The remaining urine sample was centrifuged at 3,000 rpm for 5 min, excess supernatant was removed, and the pellet was cultured on one half of an Ashdown's agar plate.

Samples of pus and respiratory secretions were serially diluted prior to spread plating on Ashdown's agar plates to obtain single colonies. Throat and wound swabs were streaked onto an Ashdown's agar plate. In addition, these specimens were cultured using enrichment broth, as previously described (250). All agar plates were examined daily for clinical diagnostic purposes, but were maintained at 37°C in air for 4 days before colonies were selected for genotyping.

Colonies of presumptive *B. pseudomallei* were initially identified on the basis of colony morphotype. This included the characteristic colony morphology (purple, flat, dry and wrinkled) together with 6 additional colony morphotypes, as described previously (24). Colonies suspected to be *B. pseudomallei* were tested using the oxidase test, and then confirmed using a *B. pseudomallei* specific latex agglutination test (262). Colonies on the primary plate were picked for genotyping when available for a given sample; in the event that enrichment broth was positive but primary plates were negative, colonies were picked following sub-culture. For samples with a single *B. pseudomallei* colony morphology type present, 10 colonies were picked and saved to independent freezer vials. All colonies were picked if less than 10 were present. If more than one colony morphology was present, 5 of each were picked to independent vials.

4.2.3 Bacterial genotyping.

Genotyping was performed using a combination of PFGE and MLST. All colonies were subjected to PFGE using *SpeI*. Samples from an individual patient were run on the same gel and analysed using BioNumerics (version 2.5) software (Applied Maths, Belgium). Colonies from a given patient with an identical PFGE banding pattern were regarded as genotypically indistinguishable, but isolates with one or more bands different were further examined using MLST. PFGE and MLST were performed as described in chapter 2.

4.3 Results.

A total of 215 samples were processed from 133 patients with culture-confirmed melioidosis, of whom 8 (6.0%) were less than 15 years of age, and 4 (3.0%) were from cases of recurrent melioidosis. Sample types were blood (n=73), respiratory secretions (n=54), urine (n=20), pus (n=18), throat swab (n=26) and wound swab (n=24). Mixed *B. pseudomallei* morphotypes were observed on 18 of 184 (9.8%) primary plates. Morphotype I to VII were present in 178 (96.7%), 13 (7.1%), 6 (3.3%), 4 (2.2%), 6 (3.3%), 7 (3.8%), and 3 (1.6%) specimens, respectively.

A total of 2,058 colonies were examined by PFGE. The median number of colonies tested per patient was 10 (IQR 10-20, range 1-70). Two patients had only a single colony of *B. pseudomallei* grown from the Isolator tube, and one patient had 70 colonies obtained from 7 specimens (blood, urine, respiratory secretions and 4 swabs from pustules at forehead, hip and both legs).

A total of 130 patients (97.7%) had colonies with an identical PFGE banding pattern, consistent with infection by a single strain of *B. pseudomallei*. Three patients had samples containing colonies with two different PFGE banding patterns, details of which are summarized in table 4-1. MLST was performed on a representative of each banding pattern from each sample. This demonstrated that two of the three patients were each infected with two different strains of *B. pseudomallei*, confirming polyclonal *B. pseudomallei* infection in 2/133 cases (1.5%; 95%CI, 0.2-5.3%). The third patient had multiple samples containing strains that differed by 2 bands on PFGE, which on MLST was shown to be a single clone (table 4-1). The two patients with polyclonal infection survived to hospital discharge, completed 20 weeks of oral antibiotic treatment and were well on 3 month follow up.

Table 4-1. Details of 3 patients who had isolates from primary plates with different PFGE banding patterns.

Identifier	Sample type	Colony Type	Colonies tested	PFGE pattern	MLST	Clinical features and outcome
Patient 1	Respiratory secretions	Type I	10	Pattern A, 9/10 colonies	A is ST 207	Female, 3 yrs old, presented with right parotid abscess and febrile convulsion. Survived.
				Pattern B, 1/10 colonies	B is ST 9	
				A & B differ by 12 bands		Simultaneous infection with more than one strain of <i>B. pseudomallei</i> .
Patient 2	Throat swab	Type I	10	Pattern C, 10/10 colonies	C is ST 167	Female, 55 yrs old, diabetes presented with acute pneumonia and hypotension. Survived.
				Pattern C, 9/10 colonies	D is ST 511	
				Pattern D ^a , 1/10 colonies		Simultaneous infection with more than one strain of <i>B. pseudomallei</i> .
				C & D differ by 11 bands		

Patient 3	Blood	Type I	9	Pattern D ^a , 9/9 colonies	D is ST 511	Female, 30 yrs old, presented with acute pneumonia and acute hyperglycaemia. Survived. Infection with single strain of <i>B. pseudomallei</i> .
	Throat swab	Type I	10	Pattern D, 9/10 colonies Pattern E, 1/10 colonies	E is ST 511	
	Respiratory secretions	Type I	5	Pattern D, 4/5 colonies		
		Type II	5	Pattern E, 1/5 colonies		
		Type IV	5	Pattern D, 4/5 colonies		
				Pattern E, 1/5 colonies		
			Pattern D, 5/5 colonies D & E differ by 2 bands for all samples			

^a Patients 2 and 3 were both infected with *B. pseudomallei* ST 511; they were not admitted during the same period, and contamination of the sample taken from patient 2 with a strain from patient 3 is considered highly unlikely.

4.4 Discussion.

This large prospective study of unselected patients with melioidosis has demonstrated that primary infection with more than one strain of *B. pseudomallei* is very uncommon. These findings differ considerably from those of Pitt *et al.* (176). The reason for this is not clear; laboratory contamination leading to more than one strain in a sample or freezer vial is unlikely since the PFGE patterns were not shared between isolates associated with mixed infection, although this does not rule out the possibility. Case and sample selection could lead to variable results between the two studies. Pitt *et al.* (176) reported mixed infection involving samples collected during the months of August or September of 1992 or 1993, compared with this study when recruitment was performed of consecutive patients between June and September of 2006. The distribution of sample types was also different, with 14/18 (78%) samples in the study by Pitt *et al.* representing blood cultures compared with 73/215 (34%) in this study. The possibility that *in vivo* selection of a biologically successful clone occurs following mixed infection has not been excluded, but this seems unlikely given the approach taken here of culturing all available sample types in a given patient.

It is proposed that the re-infection rate of 25% defined for patients with recurrent infection described in chapter 3 represents a true measure, and that patients who survive to hospital discharge following a first episode require counseling regarding the risk and strategies for avoidance of further, independent episodes of melioidosis.

4.5 Chapter summary.

Simultaneous infection with more than one strain of *Burkholderia pseudomallei* is uncommon in human melioidosis. A difference in disease severity was not observed between patients with single clone infection and polyclonal infection, although this calculation was underpowered as a result of the small number of patients with mixed strain infection.

Chapter 5. Genetic diversity of *B. pseudomallei* in the environment.

5.1 Chapter content.

It is possible that 'relapse' could actually represent re-infection in the event that re-infection is caused by a *B. pseudomallei* clone that is indistinguishable from that caused the primary episode. The probability of this occurring is based on the genetic diversity of *B. pseudomallei*, and in particular the diversity of strain present in the environment. Individuals are repeatedly exposed during their agricultural work to a defined, often small area of soil.

The aim of this study was to evaluate the genetic diversity and population structure of *B. pseudomallei*. This was achieved by:

1. Defining the genetic population of *B. pseudomallei* in a defined area of land in northeast Thailand.
2. Evaluating the genetic population of *B. pseudomallei* in Thailand.

5.2 Materials and Methods.

5.2.1 Study site.

Soil samples were collected during September 2005 (the rainy season) from an area of disused land measuring 237.5 m² (23.75 m x 10 m) situated to one side of road 231 in Amphoe Meung, at a distance of 8 km northeast of Sappasithiprasong Hospital, Ubon Ratchathani, northeast Thailand. This is a rural rice growing region, and road traffic is light. Cattle range through the area, although none were present on the land during sampling. The soil type was sandy loam, and was wet under foot but not flooded. The vegetation was low lying scrub and the area showed no signs of cultivation. The site included a single concrete electricity pole. A brick wall formed one boundary, running parallel to and distal from the road.

Five people undertook sampling over a 2 day period of intermittent rain. The site was initially divided into a grid system using string and wooden stakes, in which 5 x 20 spots were plotted 2.5 m apart on the vertical axis, and 1.25 m apart on the horizontal axis. The spot was referenced by letters of the alphabet (A to E for horizontal rows as viewed with back against the road, row A lying closest to road) and numerically (1 to 20, moving across from left to right on vertical axis). Each spot is hereafter termed a 'sampling point' and the specific site defined by its grid reference.

5.2.2 Soil sampling.

A hole was dug with a clean spade to a depth of approximately 30 centimetres. A clean plastic bag was placed on weighing scales and a sample of soil (100 grams) was removed from the base of the hole and placed into the bag. Each soil sample was labeled using pre-prepared stickers denoting the grid reference number. The bag was closed and stored out of direct sunlight at ambient temperature until transported to the laboratory where it was processed on the same day. The utensils used for sampling

were cleaned between each use by rinsing with bottled water to remove visible debris, followed by cleaning with 70% ethanol and air drying.

5.2.3 Soil culture and *B. pseudomallei* identification.

Soil samples were batch processed at the end of each collection day. 100 ml of sterile water was added to each bag, mixed well and left overnight to sediment. The upper layer of water was then transferred by plastic pipette to a sterile plastic container. Four aliquots of 100 μ l were spread plated onto each of 4 Ashdown's selective agar plates. A further 1 ml of the soil water sample was added to 9 ml of selective enrichment broth consisting of threonine-basal salt plus colistin (TBSS-C50 broth). This was incubated at 40°C in air for 48 h, after which 10 μ l of surface liquid was plated onto a second Ashdown's agar plate which was incubated and observed as before. Agar plates were incubated at 40°C in air and visually inspected daily for 4 days. Colonies of *B. pseudomallei* were initially identified on the basis of colony morphotype. This included the characteristic colony morphology (purple, flat, dry and wrinkled) together with 6 additional colony morphotypes, as described previously (24). Colonies suspected to be *B. pseudomallei* were tested using the oxidase test, and positive colonies confirmed as *B. pseudomallei* using the specific latex agglutination test (262).

5.2.4 Colonies and genotyping of *B. pseudomallei*.

Genotyping of *B. pseudomallei* was performed for 3 sampling points (grid reference A11, D10 and E4). These were selected at random from sampling points that gave at least 200 *B. pseudomallei* colonies on the primary Ashdown's agar plates. For each sampling spot selected, all primary colonies were picked to purity and subjected to PFGE using *SpeI*. Analysis of PFGE banding patterns for the 200 colonies at each of the three sampling points was performed using BioNumerics (version 2.5) software

(Applied Maths, Belgium). For the purposes of this study, interpretation was defined so as to be highly discriminatory. Isolates with identical PFGE banding patterns were regarded as genotypically indistinguishable, but isolates with one or more bands different were defined as a putatively different and given a different banding pattern number. One bacterial representative of each banding pattern type was further examined using MLST. PFGE and MLST were performed as described in chapter 2.

5.2.5 Measures of genetic diversity.

5.2.5.1 Genetic diversity of *B. pseudomallei* in disused land.

Genetic diversity of *B. pseudomallei* within a given sampling point was defined using Simpson's index of diversity. This describes the probability that two randomly selected bacterial cells within a sampling point will be different genotypes; 0 indicates no diversity and 1 indicates infinite diversity. Confidence intervals for Simpson's index were calculated as described previously (103). Genetic diversity between two sampling points was measured using the Morisita index of similarity. This ranges from 0 to 1; 0 indicates that no genotypes are shared between the two sampling points, and 1 indicates complete identity. Further analysis was performed to examine whether the genetic distance between isolates within a given sample was significantly different from that expected if all isolates were randomly distributed between three sites. The number of different alleles (0->7) was determined for all 19900 pairwise comparisons of the 200 colonies (strains) at each of the three sampling points. This was compared to mean values calculated from 100 random samples, each of 200 strains, drawn with replacement from the combined data set of 600 strains (all three sample sites). Statistical significance was gauged by calculating the 0.01, 0.05 and 0.95 and 0.99 percentiles from the resampled data. All analyses were calculated using Stata 9.0

(College Station, Texas, United States), except the random resampling procedure which used a Perl script.

5.2.5.2 Genetic diversity of *B. pseudomallei* in Thailand.

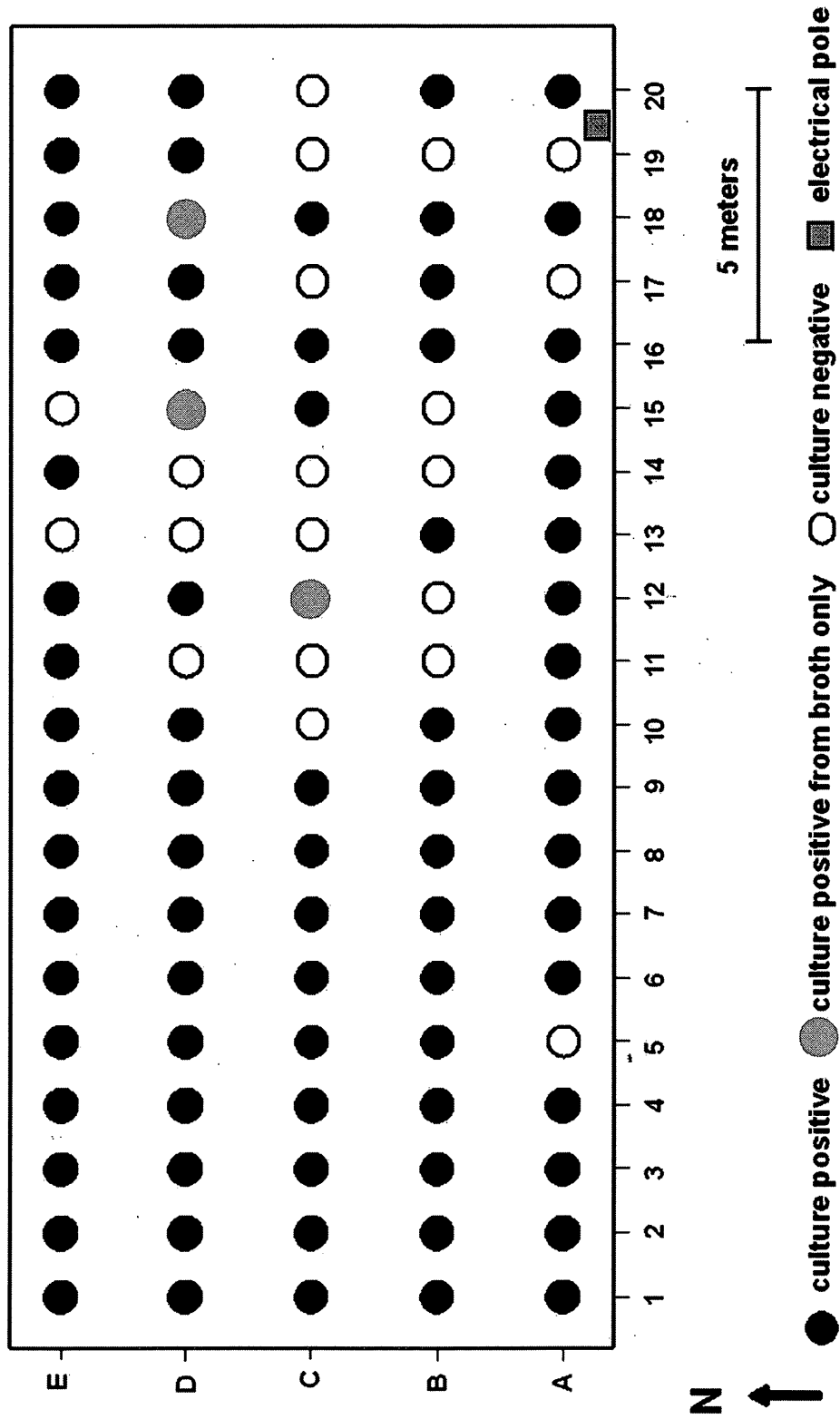
ST data were downloaded from the MLST database (<http://bpseudomallei.mlst.net>) for all isolates listed as having originated from Thailand. Diversity was defined based on the number of isolates per ST, and total number of STs resolved for this population.

5.3 Results.

5.3.1 Positivity of *B. pseudomallei* culture from soil sampled from disused land.

A total of 80 out of the 100 sampling points were culture positive for *B. pseudomallei*, of which 77 were positive from both direct plating onto Ashdown's agar and selective enrichment broth, and 3 were positive from selective enrichment broth culture alone (figure 5-1.) *B. thailandensis* was not detected.

Figure 5-1. Soil sampling within an area of disused land in northeast Thailand to determine the presence of *B. pseudomallei* in 100 spaced sampling points.



5.3.2 Genotyping of *B. pseudomallei* isolated from soil.

The genetic variability of *B. pseudomallei* was defined and compared within and between sampling points by genotyping 200 colonies at each of three positive points (A11, D10 and E4, see figure 5-1). PFGE of 600 individual primary colonies revealed 12 PFGE banding pattern types. MLST of a single random isolate of each of the 12 PFGE types revealed 9 STs (table 5-1). Figure 5-2 shows the PFGE banding patterns.

Figure 5-2. Twelve different PFGE banding patterns identified for soil isolates.

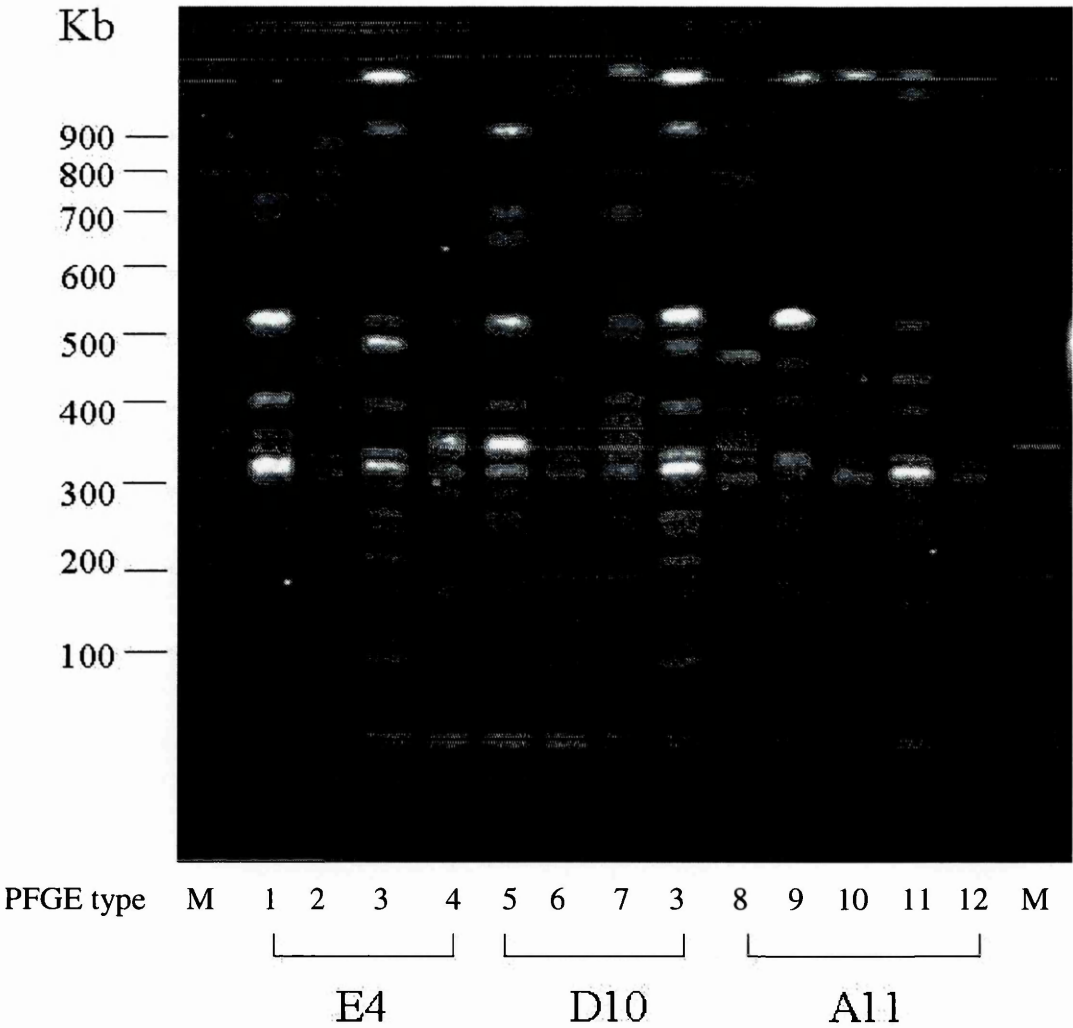


Table 5-1. Results of PFGE and MLST for soil isolates.

PFGE Type	ST	MLST profile						
		<i>ace</i>	<i>gltB</i>	<i>gmhD</i>	<i>lepA</i>	<i>lipA</i>	<i>narK</i>	<i>ndh</i>
1	ST 424	4	12	10	4	8	3	1
2	ST 177	1	1	4	3	1	3	1
3	ST 176	3	1	4	1	1	3	1
4	ST 185	1	4	2	2	1	4	1
5	ST 33	1	4	12	1	1	2	1
6, 11, 12	ST 60	3	1	12	1	1	3	1
7	ST 163	3	2	2	1	1	4	1
8, 10	ST 93	1	1	2	1	1	4	1
9	ST 304	1	1	5	1	1	4	1

5.3.3 Genetic diversity of *B. pseudomallei* in soil.

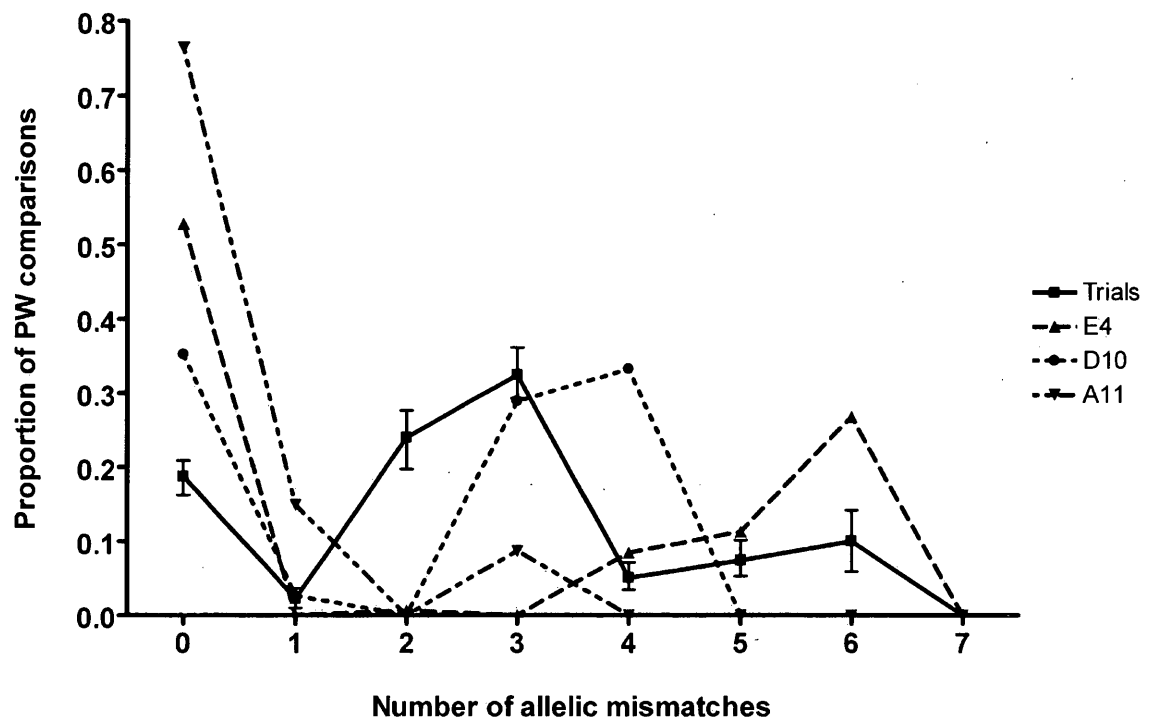
Table 5-2 shows the breakdown of STs within each of the three sampling points. D10 and E4 each contained four STs and A11 contained three STs. Although the distance between the 3 sampling points was low (7.6, 7.9 and 13.3 meters for A11-D10, D10-E4, and A11-E4, respectively), only two STs were present in more than one sampling point (E4/D10; ST176, and D10/A11; ST60); no STs were common to E4 and A11, and no STs were detected in all three points. This strong separation between sites is reflected in low values of the Morisita index (table 5-2). Furthermore, each site was characterized by the following predominant genotypes, each of which was restricted to a single site: ST93 in A11 (87%), ST163 in D10 (51.5%), and ST185 in E4 (70%). Simpson's index of diversity ranged from 0.24-0.65 (table 5-2).

Table 5-2. Genotyping results for 200 colonies of *B. pseudomallei* from each of three independent sampling points.

ST	Sampling points					
	E4		D10		A11	
ST 424	38	(19%)				
ST 177	12	(6%)				
ST 176	10	(5%)	29	(14.5%)		
ST 185	140	(70%)				
ST 33			50	(25%)		
ST 60			18	(9%)	9	(4.5%)
ST 163			103	(51.5%)		
ST 93					174	(87%)
ST 304					17	(8.5%)
Simpson's index of diversity (95% CI)	0.47 (0.40-0.54)		0.65 (0.60-0.69)		0.24 (0.16-0.31)	
Morisita Index of similarity						
- compared to E4	-		0.02		0.00	
- compared to D10	-		-		0.01	

The finding of very limited overlap between the sites was further examined by comparing the average pairwise distance (in terms of allelic mismatches) between isolates within each site to that expected if the three sample sites were combined as a single population. Figure 5.3 shows the proportion of all 19,900 $((200 * 199) / 2)$ pairwise comparisons showing 0, 1, 2 ... 7 allelic mismatches for each of the seven MLST loci for 200 colonies (strains) examined at each of three independent sampling points. This was compared to randomised data derived from the mean values for 100 random samples of 200 strains resampled with replacement from the combined data set of 600 strains from all three sites. All three sampling sites showed a significantly greater proportion of identical pairs (i.e. the same ST) than the randomised data ($P < 0.01$), which is consistent with localized clonal expansion. For two of the three sampling sites, there was no significant difference in the proportion of related but non-identical STs (i.e. those differing at a single locus) compared with the randomised data. The exception was site A11 where a significantly higher number of pairwise comparisons ($P < 0.01$) corresponded to a single MLST locus difference; ST304 is a single locus variant of the predominant clone ST93, being variant at *gmhD*. Comparison of the two *gmhD* alleles (allele 2 for ST93 and allele 5 for ST304) indicated two base differences (C→T position 118, and T→C position 327). The other ST at A11 is ST60; this differs at three loci from both ST93 and ST304, accounting for the small peak at three mismatches in the A11 plot.

Figure 5-3. Graph of the proportion of all pairwise comparisons showing allelic mismatches for each of 200 primary colonies (strains) examined at three independent sampling points.



* Error bars are based on the 5th and 95th percentiles of the 100 random samples.

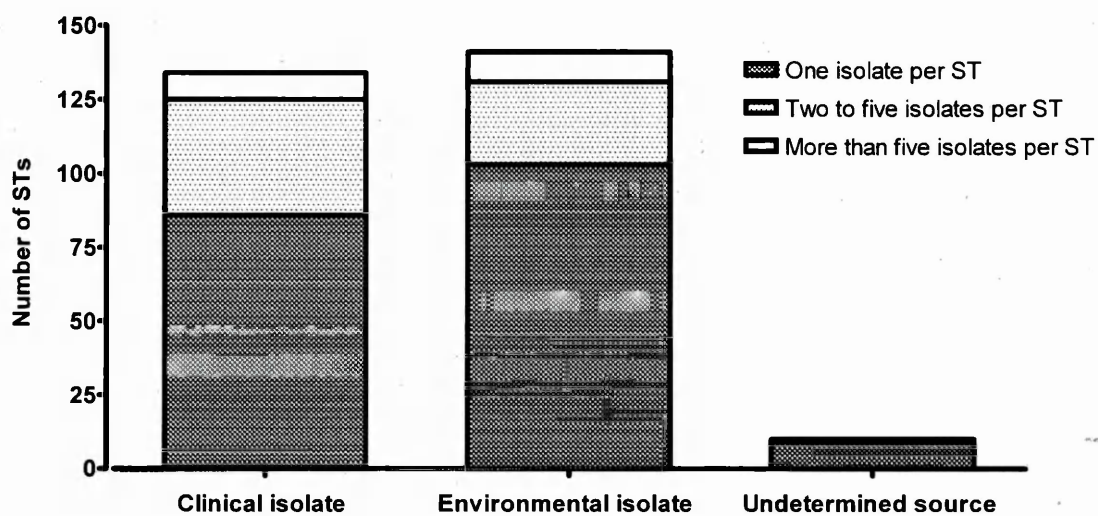
No pw comparisons (real or trial data) differ at all seven sites since the locus *ndh* is monomorphic (invariant).

Comparison of PFGE and MLST results demonstrated that two STs contained strains with variable PFGE banding pattern types. ST93 contained strains with two PFGE types (types 8 and 10), which were 12 bands different. This ST was only found in sampling site A11, in which the proportion of each banding pattern was 172/174 (99%) for type 8, and 2/174 (1%) for type 10. ST60 contained strains with three PFGE types (types 6, 11 and 12). The difference in banding patterns between these three was 6 bands (PFGE types 6 versus 11), 1 band (PFGE types 6 versus 12), and 2 bands (PFGE types 11 versus 12). ST60 was recovered from two sampling points (A11 and D10, 7.6 m apart). The proportion of each PFGE type identified at the two sampling points was as follows: sampling point A11 (total 9 colonies), type 11 (8 colonies) and type 12 (1 colony); sampling point D10, all 18 colonies were type 6. The number of PFGE bands different between the single locus variants ST304 (type 9) and ST93 (types 8 and 10) were: type 9 vs. type 8, 18 bands; type 9 vs. type 10, 10 bands.

5.3.4 Genetic diversity of *B. pseudomallei* in Thailand.

MLST revealed a high level of diversity for *B. pseudomallei* isolates listed in the public MLST database as having been isolated in Thailand from both clinical and environmental samples. There were 285 different STs for the 565 *B. pseudomallei* isolates in the database, as of 7th August 2007. Of these, 198 STs (69%) are represented by single isolates, and 87 STs (31%) included more than 1 isolate (range, 2 to 21). This high diversity was observed for both clinical isolates and environmental isolates when considered separately (figure 5-4). Simpson's index of diversity was 0.92 (95%CI, 0.92-0.93) for clinical isolates and 0.96 (95%CI, 0.95-0.96) for environmental isolates.

Figure 5-4. Bar graph representing the number of isolates per ST found in clinical isolates, environmental isolates, and isolates with undetermined source originating from Thailand.



* Data was from <http://bpseudomallei.mlst.net>.

5.4 Discussion.

This study has demonstrated marked geographic structuring of *B. pseudomallei* genotypes in disused land. A high genetic diversity was also demonstrated for all Thai *B. pseudomallei* isolates listed in the MLST database. Simpson's index of diversity of around 0.95 indicates that the probability that a given individual will develop primary infection and re-infection with isolates sharing the same ST is extremely remote. This may become more likely if host genetic susceptibility were to select for specific bacterial clones, but there is no evidence that this is the case for melioidosis. Extreme genetic diversity over a small area of the environment suggests that an individual who repeatedly works within a restricted area of land is nonetheless exposed to a genetically diverse *B. pseudomallei* population. Taken together, these findings support the validity of genotyping in order to delineate relapse and re-infection in patients with recurrent melioidosis.

The dramatic differences in genotype frequency over such small distances are striking, but difficult to interpret. One explanation is that the numerically dominant ST at each sampling point represents a strain with superior biological fitness compared with STs present as a minority of the population. This could relate to factors such as soil type or pH, or competition with other microbial species. This would assume that adjacent foci of soil have variable microenvironments; it seems unlikely that nearby sampling points within a confined area of disused land would differ so dramatically. An alternative possibility is that of local competition between clones of *B. pseudomallei*. Soil may be subjected to events such as flooding which provides an opportunity for a given clone to migrate and become established within a specific focus. Once the clone has reached a certain threshold frequency, it could repel invaders either by the production of microbicides, through phage to which they are themselves resistant, or via other killing mechanisms. The presence of a clone as a minority

population could represent the ability of this strain to survive at a lower level, or could represent the boundary of a point of predominance in an adjacent point or focus.

This study also provides evidence for microevolution of *B. pseudomallei* in soil. PFGE is a more sensitive marker of genetic change than MLST, since alterations in banding pattern arise due to genetic events that alter the presence or absence of restriction sites for the enzyme used, while housekeeping genes are more highly conserved. Two of the nine STs contained strains with variable PFGE banding pattern types. These changes may represent microevolution within the sampling site rather than importation of the same ST with a different banding pattern, based on the low probability of co-localization of two strains with the same ST on a background of marked genetic diversity of the overall population. This is consistent with the finding that genomic islands constitute ~6% of the *B. pseudomallei* K96243 genome (109). Furthermore, comparison of the whole genome sequence of *B. pseudomallei* and *B. mallei* indicates the capacity for genomic rearrangement and gene loss by two species that are highly related by MLST (165). These findings are consistent with a dynamic genome that is evolving through the movement of genomic islands and rearrangements such as inversions, indels and the movement of insertion sequences.

The co-existence of a single locus variant (ST304) of ST93 in a single soil sample can be explained by *in situ* microevolution or by a chance association. ST93 and ST304 have both been isolated previously in northeast Thailand. Our unit recovered ST93 from the environment in 1990, 1998 and 1999, each from different sampling sites situated along road 212 which runs northwest from the town of Ubon Ratchathani. The MLST database (www.mlst.net) contains a fourth ST93 isolate that was associated with human disease in Thailand in 1998. Our unit have also recovered ST304 from two different patients with melioidosis presenting to a hospital in northeast Thailand in 1999. However, the marked genetic diversity of *B. pseudomallei* suggests

that the probability of these two STs co-existing by chance in the same soil sample is extremely low.

An important potential confounder of this study is that the proportion of each ST is obtained after the soil sample has been prepared and then grown using rich media. This raises the possibility that some STs are more adapted to survival or growth after the addition of distilled water during sample preparation, or in moving to the surface water layer which is then removed for culture. It is also possible that some STs are more likely to grow on laboratory media than others, and that some STs are viable but non-culturable under the conditions used. Resolution of these issues will require the direct and immediate application of molecular tools to soil samples, and comparison of genotypes with those obtained using conventional culture and existing soil preparation methods.

These findings have important implications for future genotyping studies. Soil sampling at a single location will fail to identify the genotypes present even a few meters away. Furthermore, the dominance of a single ST at a given site indicates that extra sampling effort is required to detect rare genotypes which may be present. For example, the characterization of approximately 50 colonies from any single site would provide an 85% probability of detecting a genotype present at the site at a frequency of 2%. This is based on the exact 95% binomial confidence interval for ST60 at sampling point A11, which was present in the lowest number.

These findings also have implications for future studies in which PCR is performed directly on soil extracts to detect *B. pseudomallei*. The amplification product may be mixed, and DNA from strains present at low copy number is unlikely to undergo amplification. Thus, PCR represents acceptable technology if the objective is to determine *B. pseudomallei* presence or absence, but would be inappropriate if PCR is used as the basis for subsequent genotyping unless multiple independent amplicons are

evaluated. The problem this then poses is how a genotyping scheme could be devised based on the sequence of a single PCR product.

This study has investigated *B. pseudomallei* in soil taken from an area of disused land. This is in contrast to many previous studies in Thailand which were conducted in rice paddies. The basis for this choice was to examine an environment free of external influences such as chemical fertilizers and pesticides, together with the effect of ploughing, planting, burning of rice stubble and the presence of rice plants. However, most disease is probably acquired in rice paddies; further studies are required to compare and contrast the findings reported here with those from a rice paddy in the same region.

5.5 Chapter summary.

A study was performed to determine the presence and genotypes of *B. pseudomallei* in soil sampled from disused land in northeast Thailand. *B. pseudomallei* was present on direct culture of 77/100 sampling points. Genotyping of 200 primary plate colonies from 3 independent sampling points was performed using a combination of PFGE and MLST. Twelve PFGE types and 9 STs were identified, the majority of which were present at only a single sampling point. Despite the proximity of the sampling points, each of the three samples was characterized by the localized expansion of a single clone, corresponding to STs 185, 163 and 93. High genetic diversity of *B. pseudomallei* was also observed for isolates originating from Thailand that are listed in the public MLST database. Extreme structuring of genotypic frequency in soil suggests that the probability of an individual becoming re-infected with the same clone as that causing the primary episode is extremely remote. This supports the use of genotyping to differentiate between relapse and re-infection in patients presenting with recurrent melioidosis.

Chapter 6. Risk factors for recurrent melioidosis in northeast Thailand.

6.1 Chapter content.

Risk factors for recurrence have been defined previously and include severity of initial disease, antimicrobial drugs used and treatment duration (27;29;42;184). These studies have largely treated recurrence as a homogenous condition, with cases being assumed to have resulted from relapse of a persistent focus of infection.

The aim of this study was to re-evaluate relapse and re-infection separately to define their individual risk factors. This was achieved by evaluating a cohort of patients with culture-confirmed melioidosis who presented to Sappasithiprasong Hospital during a 19-year period to define risk factors for relapse and re-infection. Specifically:

1. Clinical factors and antimicrobial treatment in patients with recurrent disease due to relapse confirmed by bacterial genotyping were compared using a time-varying Cox proportional hazard model to determine specific risk factors.
2. The cohort was re-evaluated to determine risk factors for re-infection.

6.2 Materials and Methods.

6.2.1 Patients.

Study patients were adults (≥ 15 years) with culture-confirmed melioidosis who presented to Sappasithiprasong Hospital, Ubon Ratchathani, northeast Thailand between June 1986 and July 2004. All patients were followed up until July 2005. Patients with suspected melioidosis were identified and managed as described in chapter 3. Recurrent infection was detected from the medical history and via cross-referencing within the patient database. Ethical permission was obtained from the Ethical and Scientific review sub-committee of the Thai Ministry of Public Health.

6.2.2 Definitions.

Predisposing factors for melioidosis defined here were diabetes mellitus, renal calculi, chronic renal failure, thalassemia, connective tissue disease requiring steroid therapy or other immunosuppressive treatment, alcoholism, chronic liver disease, malignancy and traumatic injury. Diabetes mellitus was defined as either pre-existing, a new diagnosis as defined by the American Diabetes Association criteria (2), or acute hyperglycaemia (random plasma glucose > 11.1 mmol/L) after recovery requiring hypoglycaemic drug treatment. Impaired renal function was defined as an estimated glomerular filtration rate below 60 mL/min/1.73 m² during admission. Glomerular filtration rate was estimated using an abbreviated form of the Modification of Diet in Renal Disease study equation (4). Melioidosis was classified as bacteraemic (blood culture positive plus a single or no identifiable focus of infection), disseminated (blood culture positive plus > 1 non-contiguous foci of infection), localized (single focus of infection and blood culture negative), or multifocal (> 1 non-contiguous foci of infection and blood culture negative). Recurrent melioidosis was defined as described in chapter 3.

6.2.3 Treatment.

Standard of care throughout the study period was in-patient intravenous antimicrobial therapy followed by a prolonged course of oral drugs. Many patients were included in prospective studies of antimicrobial chemotherapy and as a result the regimens used varied over time.

In 1986, the recommended parenteral treatment was a four-drug combination of chloramphenicol, trimethoprim, sulphamethoxazole and doxycycline. A trial conducted between September 1986 and July 1988 comparing this regimen to intravenous ceftazidime (255), resulted in a change in first-line parenteral treatment to ceftazidime. A trial conducted between January 1989 and October 1992 compared intravenous ceftazidime and intravenous AMC (224), and a trial conducted between August 1994 and November 1997 compared intravenous ceftazidime and imipenem (201). Patients outside clinical trials were treated with ceftazidime. Parenteral therapy was changed if the clinician considered that treatment was failing, or if there was a significant adverse reaction to the drug. Oral treatment was substituted when the patient showed sufficient clinical improvement. Timing of the switch to oral antimicrobial drugs was based on clinical judgment plus local guidelines that parenteral therapy should be given for at least 7 days (1986 to 1993), or for at least 10 days (1994 onwards).

Standard oral treatment was either a combination of chloramphenicol, TMP-SMX and doxycycline ('four-drug regimen'), TMP-SMX plus doxycycline ('three-drug regimen'), or AMC. A study was conducted between June 1986 and August 1989 of the efficacy of oral AMC (221), and an open randomised trial comparing oral AMC and the four-drug regimen was conducted between June 1989 and October 1992 (184). Oral fluoroquinolones were evaluated as a single agent between June 1991 and March 1995 (30). An open randomised trial conducted between October 1994 and August 1997 compared oral doxycycline alone and the four-drug regimen (27), and a trial conducted

between August 1997 and July 1998 compared oral ciprofloxacin plus azithromycin with the three-drug regimen (42). A trial comparing the four-drug versus three-drug regimen was conducted between July 1998 and October 2002 (26). Between 1986 and 1989, guidelines for oral therapy were to use the four-drug regimen for at least 6 weeks. In June 1989, the duration of oral therapy was extended to 12-20 weeks. Side effects were common with the four and three drug regimens, and AMC was used during the study period as an alternative or following drug intolerance, and was first-line treatment for children or pregnant women.

6.2.4 Follow-up and outcome.

Patients who survived to discharge entered a programme of lifelong follow up by a senior clinician and collaborator working at Sappasithiprasong Hospital (W. Chaowagul). Following discharge, patients were seen every 2-4 weeks until completion of oral treatment, and every 3-12 months thereafter. Patients who failed to attend clinic were assumed to have ceased their medication at last follow up. Follow up data to July 2005 was included in this study.

6.2.5 Data analysis.

All analyses were performed using the statistical software Stata 9.0 (College Station, Texas, United States). The analysis was predominantly episode-based, although some of the univariate descriptive statistics presented are patient-based. Analyses were based on time-to-event with time measured from the start of oral antimicrobial treatment, irrespective of whether they received intravenous therapy. Events were defined as the clinical onset of culture-confirmed recurrent infection, death from other causes, or last follow-up. Periods of parenteral treatment for clinical recurrences were treated as gaps in observation. When a patient with culture-confirmed

recurrent melioidosis survived and started oral treatment, this was treated for analysis purposes as a new episode of observation. The observation time began at zero for each recurrent episode, and the analysis was stratified by the number of previous genotype-proven relapses (as opposed to re-infections). The primary analysis aimed to determine the risk factors associated with relapse. This analysis was censored for re-infection, death from causes other than culture-confirmed melioidosis, and loss to follow-up. The secondary analysis was performed to assess risk factors for re-infection, and censored for relapse, death from other causes, and loss to follow-up. Non-informative censoring was assumed, as the reasons for censoring were considered unlikely to be associated with either relapse or re-infection.

The 28 independent variables (potential risk factors) considered included patient demographics, presence of pre-morbid conditions, body sites involved in the infective process, and antimicrobial treatment given. Log-rank tests and univariate stratified Cox regression analysis were used to compare the association between each independent variable and the two dependent variables. Time-to-event outcome was assessed using Kaplan-Meier survival analysis, and the Wilcoxon test was used for variable comparison when appropriate. Stratified multivariate Cox proportional hazard models were used to adjust for interactions between factors for relapse and re-infection, respectively. Recurrence on treatment and duration of oral treatment were also modeled as time varying covariates. The final Cox proportional hazard models were developed using purposeful selection and significant continuous variables analyzed using the methods described by Hosmer and Lemeshow (114).

6.3 Results.

6.3.1 Patients.

A total of 2,517 patients with a first episode of culture-confirmed melioidosis were recruited during the 19-year study period, of which 1,229 (49%) died during admission or were taken home to die. Of those who survived, 921 (72%) patients presented to follow up clinic at least once and were eligible for inclusion in this analysis. Patient age for the 921 patients ranged from 15 to 80 years (median 48 years; IQR 38 to 58 years), and 60% were males. One or more predisposing factors were documented for 697 of 921 (76%) patients. Diabetes mellitus was the most common underlying condition (524/921 (57%)). Impaired renal function was present in 572/921 (62%) patients. The majority of patients were admitted and given parenteral treatment, with only 15 (2%) being treated in the out-patient department with oral therapy alone. Localized disease was the most common presentation (48%), followed in frequency by bacteraemic (23%), disseminated (18%) and multifocal (11%) presentations. The median number of body sites involved was 1 (IQR 1 to 2; range 0 to 6). The four-drug regimen, AMC regimen and the three-drug regimen were initially prescribed for oral eradication therapy in 266 (29%), 248 (27%), and 196 (21%) patients, respectively.

6.3.2 Follow-up and outcome.

Total duration of follow-up was 29,750 patient-months. Median duration of oral treatment with any regimen was 18 weeks (IQR 9 to 21 weeks; range 0-244 weeks). Median duration of follow-up for patients without recurrence was 72 weeks (IQR 25 to 186 weeks; range 1 to 908 weeks). A total of 167 episodes of culture-confirmed recurrent melioidosis occurred in 147 of 921 (16%) patients. Of these, 122 (73%) paired strains were available for genotyping, while one or more *B. pseudomallei* isolates were not available for typing for 32 patients with recurrent infection. These 32

patients were excluded from further analysis since it was not possible to distinguish accurately between relapse and re-infection, giving a total of 889 patients who went forward for further analysis. There was no significant difference in demographics and clinical presentation between the group of relapse patients without paired samples and those for whom paired samples were available (data not shown). It was considered unlikely that excluding these patients would bias determination of factors associated with relapse and re-infection, though it may lead to underestimates of the size of the associated absolute risks or hazards.

Of the 122 recurrent melioidosis cases, 92 (75%) cases in 86 patients had relapse as defined by genotyping. Four of these patients relapsed twice and 1 patient relapsed three times. The other 30 (25%) episodes were re-infections as defined by genotyping. One patient had re-infection after completing treatment for an episode of relapse. Median time to relapse was 26 weeks (IQR 10 to 72 weeks; range 1 to 454 weeks), while median time to re-infection was 111 weeks (IQR 59 to 164 weeks; range 1 to 363 weeks) ($P<0.001$).

A total of 85 people died during the follow up period. Of these, 29 patients died as a result of recurrent melioidosis; 19 were in the relapse group (1 died during a second relapse), and 10 were in the re-infection group. The mortality rate associated with the first recurrent episode (relapse and re-infection combined) was 24% (28/115 patients; 95%CI, 17%-33%). This was lower than the mortality rate of 49% (95%CI, 47%-51%) for primary melioidosis in the study cases. There was no significant difference in mortality between the relapse and re-infection groups (21% versus 34%; $P=0.14$). Causes of death in the remaining 56 patients were: clinically suspected culture-negative melioidosis (19), hepatobiliary malignancy (3), chronic renal disease (2), leukaemia (2), liver cirrhosis (2), gut obstruction (1), heart disease (1), ischaemic stroke (1), nosocomial infection (1), HIV infection (1) and unknown cause (23).

6.3.3 Risk factors for relapse.

On univariate analysis, patients with renal calculi and those with a positive blood or urine culture had a greater risk of relapse (table 6-1). The distribution of infection affected risk of relapse; using localized melioidosis as the comparator, patients with multifocal, bacteraemic and disseminated melioidosis were more likely to relapse. Individual organ involvement was not associated with outcome.

In an analysis of the effects of each parenteral antibiotic, choice of drug did not affect the risk of relapse (table 6-2). Furthermore, the duration of effective intravenous therapy (classed as ceftazidime, a carbapenem drug or AMC) was not related to risk of subsequent relapse.

The first oral drug regimen used was associated with rate of relapse ($P<0.001$), but not re-infection ($P=0.07$) (table 6-2). A Kaplan-Meier graph for time to relapse was constructed to compare different first oral antimicrobial regimens (figure 6-1). Relapse was not significantly different between the standard three-drug and four-drug regimens ($P=0.42$, Wilcoxon test), but AMC and other regimens (fluoroquinolone-based regimens, doxycycline alone, and TMP-SMX alone) were associated with an increased risk of relapse. AMC was significantly associated with relapse compared to the standard three-drug and four-drug regimen ($P=0.01$ & $P=0.001$, respectively), and negatively associated with relapse compared to the group containing all other regimens ($P=0.003$). Duration of standard oral treatment was highly negatively associated with risk of relapse (HR 0.71, $P<0.001$); the hazard ratio decreasing by 29% for every 4-week increase in duration of standard oral treatment (four drug regimen, three drug regimen or AMC).

Table 6-1. Risk factors for relapse and re-infection following a first episode of melioidosis for 889 patients.

Factor	No recurrence (N=774)	Relapse (N=86)			Re-infected (N=30) ^a		
	Number (%)	Number (%)	HR (95% CI)	P-value	Number (%)	HR (95% CI)	P-value
Age (yr) (median, IQR, range)	48 (38-58, 15-80)	49 (41-58, 21-72)	1.01 (0.99-1.03)	0.22	46 (39-51, 17-68)	0.99 (0.97-1.02)	0.70
Male	448 (58%)	57 (66%)	1.51 (0.98-2.32)	0.06	20 (67%)	1.83 (0.86-3.92)	0.12
Rice farmer	636 (82%)	76 (88%)	1.41 (0.75-2.66)	0.28	26 (87%)	1.20 (0.42-3.46)	0.73
Admitted to hospital	761 (98%)	85 (99%)	1.76 (0.25-12.7)	0.57	30 (100%)	-	1.0
Admitted before 1990 ^b	84 (11%)	15 (17%)	1.36 (0.78-2.38)	0.28	3 (10%)	0.64 (0.19-2.14)	0.47
Diabetes mellitus	435 (56%)	52 (60%)	0.96 (0.63-1.46)	0.86	19 (63%)	1.09 (0.51-2.33)	0.83
Renal calculi	69 (9%)	14 (16%)	1.73 (1.00-2.99)	0.05	3 (10%)	0.91 (0.27-2.99)	0.87
Thalassemia	24 (3%)	6 (7%)	1.68 (0.73-3.87)	0.22	2 (7%)	1.72 (0.39-7.48)	0.47
Estimated GFR ^c (ml/min/1.73m ²) (median, IQR, range)	49 (31-71, 3- 274)	48 (28-65, 4-120)	1.00 (0.99-1.01)	0.85	30 (26-58, 4-94)	0.99 (0.97-1.00)	0.13
Distribution:							
- Localized	392 (51%)	28 (33%)	1.0		11 (36%)	1.0	
- Multifocal	74 (10%)	14 (16%)	2.21 (1.17-4.16)	0.02	6 (20%)	2.25 (0.79-6.40)	0.51
- Bacteraemic	174 (22%)	26 (30%)	1.92 (1.15-3.21)		8 (27%)	1.44 (0.59-3.52)	
- Disseminated	134 (17%)	18 (21%)	1.75 (0.99-3.10)		5 (17%)	1.09 (0.38-3.11)	

Solid organs involved ^d (Median, IQR, range)	1 (1-2, 0-6)	1 (1-3, 0-6)	1.17 (0.98-1.39)	0.08	1 (1-2, 0-5)	0.93 (0.65-1.34)	0.71
Blood culture positive ^e	308 (40%)	44 (51%)	1.53 (1.02-2.31)	0.04	13 (43%)	1.08 (0.52-2.22)	0.84
Urine culture positive ^e	67 (9%)	13 (15%)	2.06 (1.18-3.59)	0.01	4 (13%)	1.75 (0.61-5.03)	0.30
Sputum culture positive ^e	205 (26%)	20 (23%)	0.83 (0.50-1.36)	0.45	11 (37%)	1.80 (0.85-3.79)	0.12
Throat swab culture positive ^e	173 (22%)	17 (20%)	0.89 (0.53-1.51)	0.67	7 (23%)	1.27 (0.54-2.97)	0.59
Pneumonia ^f	311 (40%)	34 (40%)	0.95 (0.62-1.45)	0.81	16 (53%)	1.87 (0.91-3.83)	0.09
Liver abscess	181 (23%)	28 (33%)	1.36 (0.87-2.12)	0.18	9 (30%)	1.25 (0.57-2.73)	0.58
Splenic abscess	194 (25%)	31 (36%)	1.42 (0.92-2.18)	0.11	10 (33%)	1.05 (0.48-2.29)	0.90
Arthritis	83 (11%)	8 (9%)	0.81 (0.39-1.67)	0.57	1 (3%)	0.30 (0.04-2.20)	0.24
Osteomyelitis	14 (2%)	2 (2%)	0.81 (0.20-3.33)	0.77	0	-	1.0

^a One patient had re-infection after relapse.

^b In 1990 the guidelines for duration of oral treatment was changed from at least 6 weeks to 12-20 weeks

^c Estimated glomerular filtration rate = $186 \times (S_{Cr})^{-1.154} \times (Age)^{-0.203} \times (0.742 \text{ if female})$

^d Organ involvement and/or positive culture from any site, except blood

^e Culture positive for *B. pseudomallei*

^f Pneumonia was defined as the presence of clinical features plus radiographic changes and/or sputum culture positive for *B. pseudomallei*.

Table 6-2. Effect of antimicrobial treatment for first episode of melioidosis on risk of relapse and re-infection for 889 patients.

Factor	No recurrence (N=774)		Relapse (N=86)		Re-infected (N=30)		
	Number (%)	Number (%)	Hazard ratio (95% CI)	P value	Number (%)	Hazard ratio (95% CI)	P value
Parenteral treatment regimen							
Ceftazidime	610 (79%)	64 (74%)	0.85 (0.53-1.36)	0.49	22 (73%)	0.85 (0.38-1.92)	0.70
Amoxycillin-clavulanic acid	170 (22%)	16 (19%)	0.81 (0.48-1.37)	0.43	7 (23%)	1.08 (0.46-2.52)	0.86
Carbapenem drug	76 (10%)	9 (10%)	0.89 (0.45-1.78)	0.75	6 (20%)	2.07 (0.85-5.07)	0.11
Duration of effective intravenous treatment							
0 – 6 days	150 (19%)	17 (20%)	0.98 (0.96-1.00)	0.12	2 (7%)	1.01 (0.98-1.04)	0.45
7 – 10 days	138 (18%)	19 (22%)			8 (26%)		
11 – 14 days	171 (22%)	21 (24%)			5 (17%)		
≥ 15 days	315 (41%)	29 (34%)			15 (50%)		
First oral treatment regimen							
3 drug regimen ^a	186 (24%)	7 (8%)	1.0	<0.001	2 (7%)	1.0	0.07
4 drug regimen ^b	242 (31%)	9 (10%)	0.71 (0.29-1.72)		7 (23%)	1.45 (0.30-7.06)	
Amoxycillin-clavulanic acid	204 (27%)	22 (26%)	1.84 (0.85-4.01)		9 (30%)	2.67 (0.57-12.40)	
Other regimens ^c	142 (18%)	48 (56%)	4.72 (2.31-9.62)		12 (40%)	4.06 (0.90-18.23)	

Duration of oral treatment							
Up to 8 weeks	138 (18%)	50 (58%)	0.87 (0.78-0.98)	0.02	7 (23%)	1.03 (0.95-1.10)	0.50
>8 – 12 weeks	58 (8%)	8 (9%)			2 (7%)		
>12 – 16 weeks	96 (12%)	3 (4%)			4 (13%)		
>16 – 20 weeks	208 (27%)	10 (12%)			6 (20%)		
More than 20 weeks	274 (35%)	15 (17%)			11 (37%)		
Duration of standard oral regimens ^d							
Up to 8 weeks	209 (27%)	68 (79%)	0.71 (0.63-0.80)	<0.001	10 (33%)	0.93 (0.80-1.08)	0.32
>8 – 12 weeks	54 (7%)	3 (4%)			2 (7%)		
>12 – 16 weeks	91 (12%)	2 (2%)			4 (13%)		
>16 – 20 weeks	193 (25%)	3 (4%)			6 (20%)		
More than 20 weeks	227 (29%)	10 (11%)			8 (27%)		
Relapse or re-infection on treatment							
On a standard oral regimen ^d	-	-	1.0		-	1.0	
On other regimen ^c or Off treatment	-	-	5.02 (2.54-9.91)	<0.001	-	4.06 (0.40-41.4)	0.24

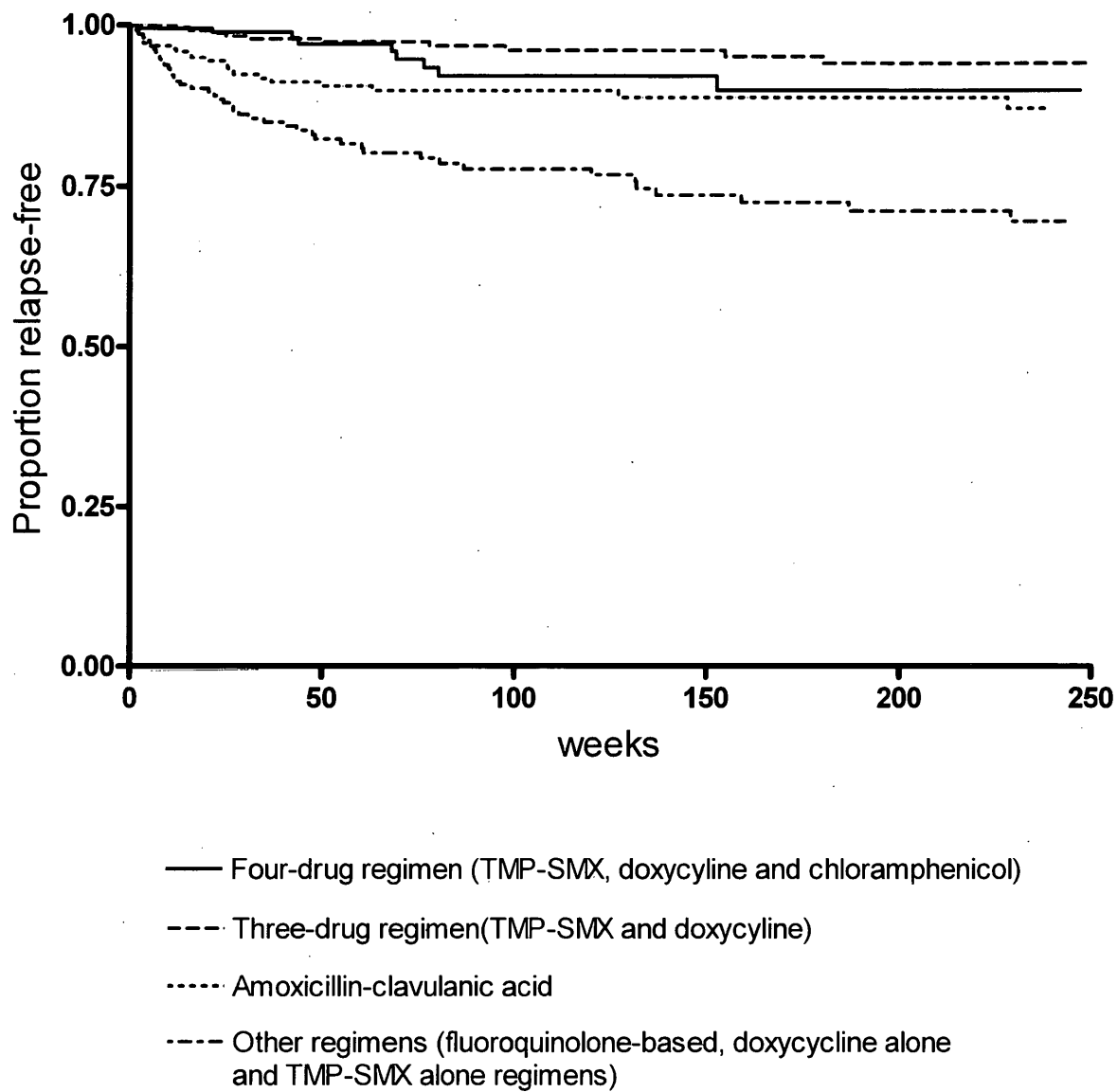
^a TMP-SMX and doxycycline

^b TMP-SMX, doxycycline and chloramphenicol

^c fluoroquinolone-based regimen, doxycycline alone and TMP-SMX alone

^d TMP-SMX, doxycycline and chloramphenicol, TMP-SMX and doxycycline, or AMC

Figure 6-1. Kaplan-Meier plot illustrating time to relapse associated with first oral antimicrobial treatment regimen for the treatment of melioidosis.



All variables were entered and developed into the multivariate model (table 6-3). Patients having a positive blood culture or multifocal distribution had a higher risk of relapse. AMC and an 'ineffective' oral treatment regimen (fluoroquinolone-based regimens and doxycycline alone) were associated with relapse. The effect of duration of standard oral treatment was evaluated by stratifying treatment duration into blocks with one-month increments (≤ 8 weeks, $>8-12$ weeks, $>12-16$ weeks, $>16-20$ weeks, and >20 weeks) (figure 6-2). Each block of treatment longer than 8 weeks compared to ≤ 8 week treatment was highly negatively associated with relapse. Risk of relapse was not significantly different between each block of treatment longer than 8 weeks (data not shown). The relationship between duration of treatment and risk of relapse was not linear, and the lowest hazard ratio was duration of treatment was between 12 to 16 weeks.

Table 6-3. Determinants of relapse in the final Cox proportional hazard model.

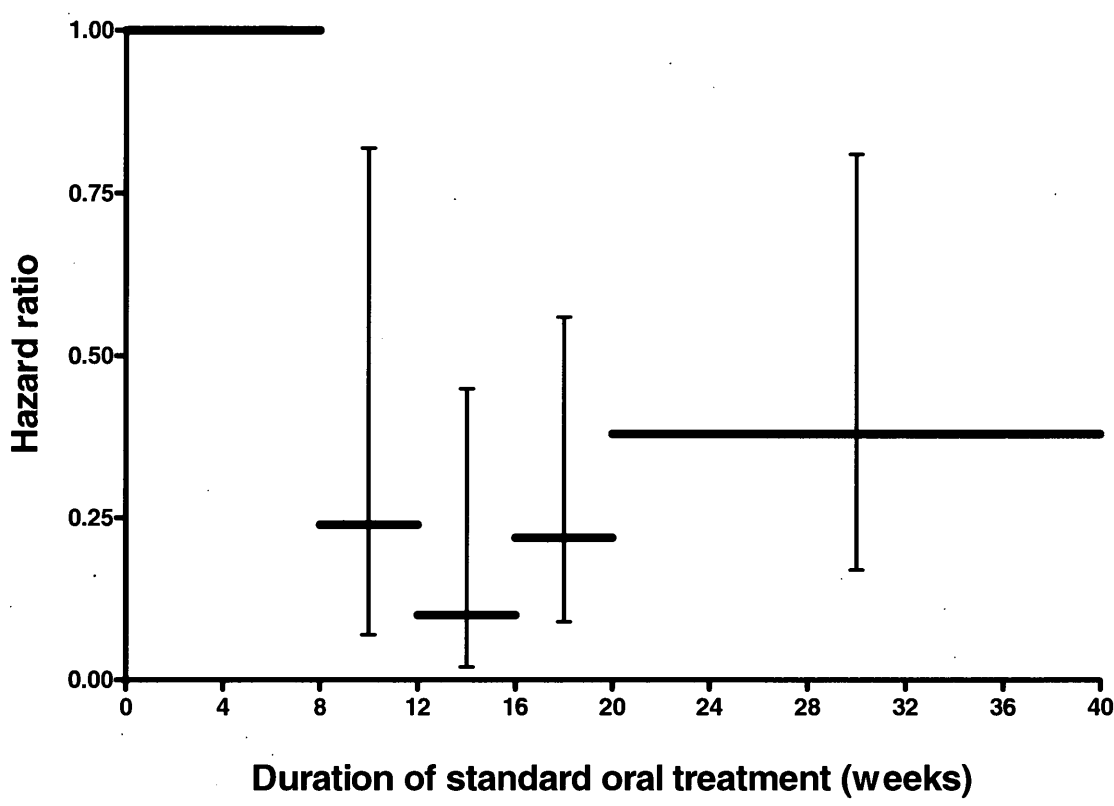
Prognostic factor	Hazard Ratio (95%CI)	<i>P</i> value
Multifocal melioidosis	2.05 (1.09-3.87)	0.03
Blood culture positive	1.84 (1.17-2.89)	0.01
First oral treatment regimen:		
- Four-drug and three-drug regimens ^a	1.0	
- Amoxycillin-clavulanic acid	2.09 (1.14-3.83)	0.02
- Other regimens ^b	3.16 (1.63-6.15)	0.001
Duration of treatment with standard regimens ^c (month)	0.87 (0.75-1.01)	0.06
Relapse on treatment		
- On a standard oral regimen ^c	1.0	0.03
- On other regimen ^b or Off treatment	2.31 (1.11-4.82)	

^a 4 drugs, TMP-SMX, doxycycline and chloramphenicol; 3 drugs, TMP-SMX and doxycycline

^b fluoroquinolone-based regimen, doxycycline alone and TMP-SMX alone

^c TMP-SMX, doxycycline and chloramphenicol, TMP-SMX and doxycycline, or AMC

Figure 6-2. Estimate of hazard ratios and 95% CIs of duration of standard oral antimicrobial treatment (TMP-SMX, doxycycline and chloramphenicol, TMP-SMX and doxycycline, or AMC) versus relapse.



* A duration of ≤ 8 weeks was used as the comparator for other treatment durations, analyzed using incremental blocks of 4 weeks (>8 -12 weeks, >12 -16 weeks and >16 -20 weeks) together with >20 weeks. Patients treated for longer than 40 weeks were excluded for the purposes of figure clarity (1 relapse patient and 32 non-relapse patients). There was no significant difference in results on comparison of data when the >40 week group were included or excluded (data not shown).

6.3.4 Risk factors for re-infection.

No factors were associated with re-infection on univariate and multivariate analyses. The incidence of re-infection in this study population after survival of a first episode of melioidosis was 1,280 per 100,000 person-years (95%CI, 895-1,831). This may be an underestimate because of the omission of re-infection cases in whom genotyping of paired samples was not possible, but is still orders of magnitude higher than previously published figures for the general population in this province of 4.4 per 100,000 person-years (95%CI, 3.8-5.0) (222).

To evaluate the possibility of bias introduced by the exclusion of recurrent patients who did not have paired *B. pseudomallei* isolates for typing, re-analysis was performed to compare results from data sets that either included or censored this group one month before recurrence occurred. There was no significant difference in outcomes for relapse and re-infection groups (data not shown).

6.4 Discussion.

The risk factors associated with relapse in this study are similar to those in the published literature (27;29;42;184). The finding that intravenous antimicrobial treatment was not associated with risk of relapse contrasts with a previous report of a higher rate of recurrence following a parenteral four-drug regimen or AMC compared with ceftazidime (29). However, the results presented here for parenteral AMC should be interpreted with caution since its use was relatively low. The current recommendation that parenteral treatment should be given for at least 10 days remains unchanged.

Choice and duration of oral antimicrobial therapy were the most important determinants of relapse. Analysis of the optimal treatment duration indicated that the lowest hazard ratio was associated with between 12 to 16 weeks of oral treatment. This is consistent with previous findings that treatment for less than 8 weeks or less than 12 weeks was associated with recurrence (26;29). Analysis of patients receiving oral treatment for >16 weeks may have been confounded by the presence of a greater proportion of patients in this group with complicated or severe disease associated with a higher risk of treatment failure and relapse. The suggested minimum duration of oral treatment of 12 to 20 weeks remains unchanged, with the actual duration being guided by clinical progression.

This study provides evidence for the efficacy of TMP-SMX plus doxycycline for oral therapy; this is consistent with an earlier report that the addition of chloramphenicol is not necessary (26). AMC was associated with an increased risk of relapse, but remains an alternative for patients with intolerance to the three-drug regimen, and continues to be used for the treatment of children and pregnant women in Thailand. Other regimens such as fluoroquinolone-based regimens and doxycycline alone were associated with a high risk of relapse, an unsurprising finding given their

lack of efficacy in previous studies (27;30;42). The number of patients who started oral treatment with TMP-SMX alone was low (n=3) and underpowered for analysis as a stand-alone group. For practical purposes, these patients were grouped into 'other regimens', and this study is not able to draw any conclusions regarding efficacy.

The definition of recurrent disease captured 5 patients that had been discharged < 2 weeks previously following treatment of a primary episode. These patients had deteriorated clinically after a clinical response, but interpretation of culture positivity in such cases is a grey area since culture can remain positive for *B. pseudomallei* for several weeks, and 'relapse' in these cases may reflect clinical fluctuations in the course of recovery from the primary episode.

This study failed to identify risk factors for re-infection; this may relate to a lack of statistical power, or the fact that relevant variables were not included. The higher rate of re-infection compared to the rate of primary infection in northeast Thailand indicates that risk factors do exist. These may relate to specific host factors or high-risk activities including occupation. Further study is required to elucidate these and determine whether preventive strategies could be implemented.

6.5 Chapter summary.

This study highlights clinical factors associated with an increased likelihood of relapse, and provides evidence for optimal oral antimicrobial therapy. There was no difference in acute outcome between relapse and re-infection groups. Multivariate analyses identified choice and duration of oral antimicrobial therapy as the most important determinants of relapse, followed by positive blood culture and multifocal distribution. Patients treated with an appropriate oral antibiotic regimen for 12 to 16 weeks had a 90% decreased risk of relapse compared with patients who were treated \leq 8 weeks. TMP-SMX plus doxycycline was an effective oral therapy. No risk factors for re-infection were identified. However, the rate of re-infection in patients who recovered from primary melioidosis was very high, indicating that prevention of re-infection is necessary.

Chapter 7. A simple scoring system to differentiate between relapse and re-infection in patients with recurrent melioidosis.

7.1 Chapter content.

Determining the cause of recurrent melioidosis is an important clinical distinction with implications for investigation and management, but the overwhelming majority of medical centres treating patients with melioidosis in Asia do not have the facilities to perform bacterial genotyping, and recurrence is usually considered to be synonymous with relapse. In addition, isolates from the primary episode may not be available if bacterial strains are not routinely frozen or if the patient had a primary episode of infection at another hospital.

The aim of this study was to define factors associated with relapse or re-infection, and to use these to develop a simple scoring system for use in resource-limited settings to predict the most probable cause of recurrent melioidosis. This was achieved by:

1. Comparing clinical factors between relapse and re-infection to determine possible predictive factors for either cause of recurrent melioidosis.
2. Developing a scoring system to differentiate between relapse and re-infection based on a multivariate logistic model with relapse/re-infection as the independent variable.

7.2 Materials and Methods.

7.2.1 Patients.

Study patients were adults (≥ 15 years) with culture-confirmed recurrent melioidosis who presented to Sappasithiprasong Hospital, Ubon Ratchathani, northeast Thailand between June 1986 and September 2005. Patients with suspected melioidosis were identified and investigated as described in chapter 3. Patients who survived the primary episode received oral eradication treatment and were followed up monthly for one year, then yearly thereafter. Oral antimicrobial regimens were as described elsewhere (chapter 6). Patients with recurrence were identified from the history, patient notes and by cross-reference with our database. Follow up data in this study was to February 2007.

7.2.2 Antimicrobial susceptibility testing.

All *B. pseudomallei* isolates were tested for susceptibility to the antimicrobial drugs used to treat melioidosis (meropenem, ceftazidime, AMC, doxycycline and trimethoprim/sulfamethoxazole (TMP-SMX)). This was performed using the disk diffusion method with the exception of TMP-SMX, which was assessed using the Etest (AB Biodisk, Solna, Sweden) (263). All isolates defined as intermediate or resistant to a given drug by disk diffusion were tested further using the Etest. Interpretative standards were based on CLSI guidelines, which lists resistance for ceftazidime, AMC, doxycycline and TMP-SMX as ≥ 32 mg/L, ≥ 32 mg/L, ≥ 16 mg/L and $\geq 4/76$ mg/L, respectively, and intermediate resistance as 16 mg/L, 16 mg/L, 8 mg/L, and N/A, respectively (57).

7.2.3 Definitions.

Diabetes mellitus and impaired renal function were defined as described in chapter 6. Hypotension was defined as a systolic blood pressure less than 90 mmHg, acute renal failure as a 50% decrease in the baseline-calculated GFR (203), and respiratory failure as the need for mechanical ventilation.

7.2.4 Statistical analysis.

All analyses were performed using the statistical software Stata 9.0 (College Station, Texas, United States). A scoring system to differentiate between relapse and re-infection was developed. The variables considered included patient demographics, presence of pre-morbid conditions, body sites involved in the primary and recurrent episode, antimicrobial treatment given for the primary episode, patterns of antimicrobial resistance for the primary and recurrent isolates and duration between primary and recurrent episode. Comparison between relapse and re-infection of each variable was performed using Fisher's exact test or the Wilcoxon-Mann-Whitney test, as appropriate. All variables associated with relapse/re-infection at $P < 0.20$ were included as independent variables in a multivariate logistic model with relapse/re-infection as the dependent variable. Variables were removed one at a time from the model if the p-value as determined by the likelihood ratio test was > 0.05 , least significant variable first. To double check that no significantly predictive variables were removed during this process, each de-selected variable was tested in turn with the final model and reintroduced into the model if $P < 0.05$ (113). Variables in the final model were used to construct a scoring system. Each statistically significant continuous variable was plotted against the predicted value and the LOWESS (locally weighted least squares) smoothing function was plotted. The shape of the curve on the distribution of values was used to suggest ranges for each variable. To assign points to

ranges, dummy variables were created for each range, and all such dummy variables were used in a multiple logistic regression analysis. The coefficient for each variable was multiplied by 10 and rounded off to the nearest integer. A total score was formulated by the sum of points from each variable for each patient, and the results plotted on a receiver-operator characteristic (ROC) curve. The Hosmer-Lemeshow goodness-of-fit test was used to evaluate the regression model, along with analysis of the area under the ROC curve.

7.3 Results.

7.3.1 Patients.

A total of 2,804 adult patients with culture-confirmed melioidosis were seen during the 19-year study period. Of these, 1,401 (50%) adult patients died during admission. Of the adults who survived, 1,001 (71%) patients presented to follow up clinic at least once. Median duration of follow-up for patients without recurrence was 65 weeks (IQR 22-179 weeks; range 1-954 weeks). A total of 194 episodes of culture-confirmed recurrent melioidosis occurred in 170 (17%) patients. Of these, 148 (76%) strains paired from the primary and recurrent episode were available for genotyping from 141 patients. Bacterial genotyping had been performed previously for 122 episodes in 115 patients as described in chapter 3, and genotyping of the remainder was performed during this study.

7.3.2 Bacterial genotyping.

Ninety-eight (66%) of the 148 recurrent melioidosis episodes in 92 (65%) of 141 patients represented relapse as defined by genotyping. Four of these patients relapsed twice and 1 patient relapsed three times. The other 50 episodes in 49 (35%) patients were caused by re-infection. One patient had re-infection after completing treatment for an episode of relapse. For the purposes of this study, only the 141 first episodes of recurrent melioidosis (92 relapse and 49 re-infection) were analyzed.

7.3.3 Antimicrobial susceptibility.

All *B. pseudomallei* isolates associated with the primary episode of recurrent infection were susceptible to ceftazidime, AMC and doxycycline, while 21/141 (15%) were resistant to TMP-SMX. All isolates associated with recurrence were susceptible to ceftazidime. Strains associated with re-infection were resistant to AMC, doxycycline

and TMP-SMX in 2% (1/49), 2% (1/49) and 16% (8/49) of cases, respectively. Strains associated with relapse were resistant to AMC, doxycycline and TMP-SMX in 1% (1/92), 1% (1/92) and 12% (11/92), respectively. The two patients with relapse associated with the development of bacterial resistance to AMC (MIC from 2 to 16 mg/L) or doxycycline (1 to 96 mg/L) received antimicrobial treatment with the respective agent for at least 8 weeks prior to relapse.

7.3.4 Specific factors associated with relapse and re-infection.

The majority of patients with re-infection presented in the rainy season, while patients with relapse presented throughout the calendar year ($P=0.002$, figure 7-1). Demographic characteristics and clinical features are shown in table 7-1. Sex and age were comparable between the two groups. Diabetes mellitus was the most common underlying condition in both relapse and re-infection. Impaired renal function was present in 42 (46%) of 92 patients with relapse and 32 of 49 (65%) patients with re-infection ($P=0.03$). Distribution of infection and organ involvement on primary infection and at time of recurrence was not different between patients with relapse and re-infection. There was no difference in severity of infection between relapse and re-infection as defined by hypotension, acute renal failure or respiratory failure ($p>0.05$ in all cases). Death occurred in 17 (18%) patients with relapse and 13 (27%) patients with re-infection ($P=0.29$).

On univariate analysis, the duration of oral antibiotic treatment for the primary episode was significantly shorter for patients with relapse than re-infection ($P<0.001$). The median time to relapse was also significantly shorter than time to re-infection (6 months versus 24 months, $P<0.001$) (figure 7-2). On multivariate analysis, significant independent predictors of re-infection were the presence of a low GFR on admission of recurrent episode and calendar period of presentation (rainy season). Short duration of

oral antimicrobial treatment for first episode of infection, and an interval between the primary infection and recurrence of less than one year were predictive for relapse (table 7-2). The area under the ROC curve for this model was 0.81 (95% CI: 0.74-0.89), and the Hosmer-Lemeshow goodness-of-fit test was not significant for lack of fit ($P=0.16$).

Table 7-1. Demographic characteristics and clinical features of patients presenting with relapse and re-infection.

Variable	Relapse (n=92)	Re-infection (n=49)	P value
Men, No. (%)	59 (64%)	29 (59%)	0.59
Age (yr) at recurrence, median (IQR)	49 (42-58)	47 (39-55)	0.25
Diabetes mellitus	58 (63%)	27 (55%)	0.37
Estimated GFR ^a on admission with recurrence, median (IQR)	60 (35-88)	42 (27-60)	0.02
Site(s) involved during recurrent infection			
Bacteraemia	43 (47%)	28 (57%)	0.29
Pneumonia	27 (29%)	17 (35%)	0.57
Liver abscess	17 (18%)	9 (18%)	1.00
Splenic abscess	14 (15%)	7 (14%)	1.00
Skin or soft tissue infection	31 (34%)	16 (33%)	1.00
Arthritis	13 (14%)	8 (16%)	0.81
Osteomyelitis	7 (8%)	1 (2%)	0.26
Complications of recurrent infection			
Hypotension	15 (16%)	11 (22%)	0.37
Acute renal failure	22 (24%)	17 (35%)	0.24
Respiratory failure	10 (11%)	7 (14%)	0.59
First oral antibiotic regimen for primary episode			
Three-drug regimen ^b	9 (10%)	6 (12%)	0.14

Four-drug regimen ^c	10 (11%)	12 (24%)	
Amoxycillin-clavulanic acid	23 (25%)	12 (24%)	
Other regimen ^d	50 (54%)	19 (39%)	
Duration of oral treatment for primary episode, weeks, median (IQR)	1 (0-5)	16 (0-21)	<0.001
Recurrence in rainy season (June to November)	44 (48%)	37 (76%)	0.002
Time to recurrence (months) median (IQR)	6 (2-17)	24 (9-45)	<0.001
Death attributable to recurrent melioidosis	17 (18%)	13 (27%)	0.29

^a mL/min per 1.73 m²,

^b Trimethoprim-sulfamethoxazole and doxycycline,

^c Trimethoprim-sulfamethoxazole, doxycycline, and chloramphenicol,

^d Fluoroquinolone-based regimen, doxycycline alone, and trimethoprim-sulfamethoxazole alone.

Table 7-2. Multivariable predictors of re-infection among patients with recurrent melioidosis

Predictor	OR (95% CI)	<i>P</i> Value
Time to recurrent melioidosis more than one year	3.56 (1.53-8.27)	0.003
Presentation in rainy season (June to November)	3.31 (1.37-8.00)	0.008
Duration of oral treatment received ^a	1.04 (1.01-1.08)	0.012
Estimated GFR on admission with recurrence (mL/min per 1.73 m ²) ^b	0.83 (0.72-0.95)	0.007

^a The OR is for a week increase of treatment with effective oral treatment regimens, including TMP-SMX and doxycycline based regimens and AMC regimen

^b The OR is for a 10 mL/min per 1.73 m² increase

Figure 7-1. Calendar month of presentation for patients with relapse or re-infection.

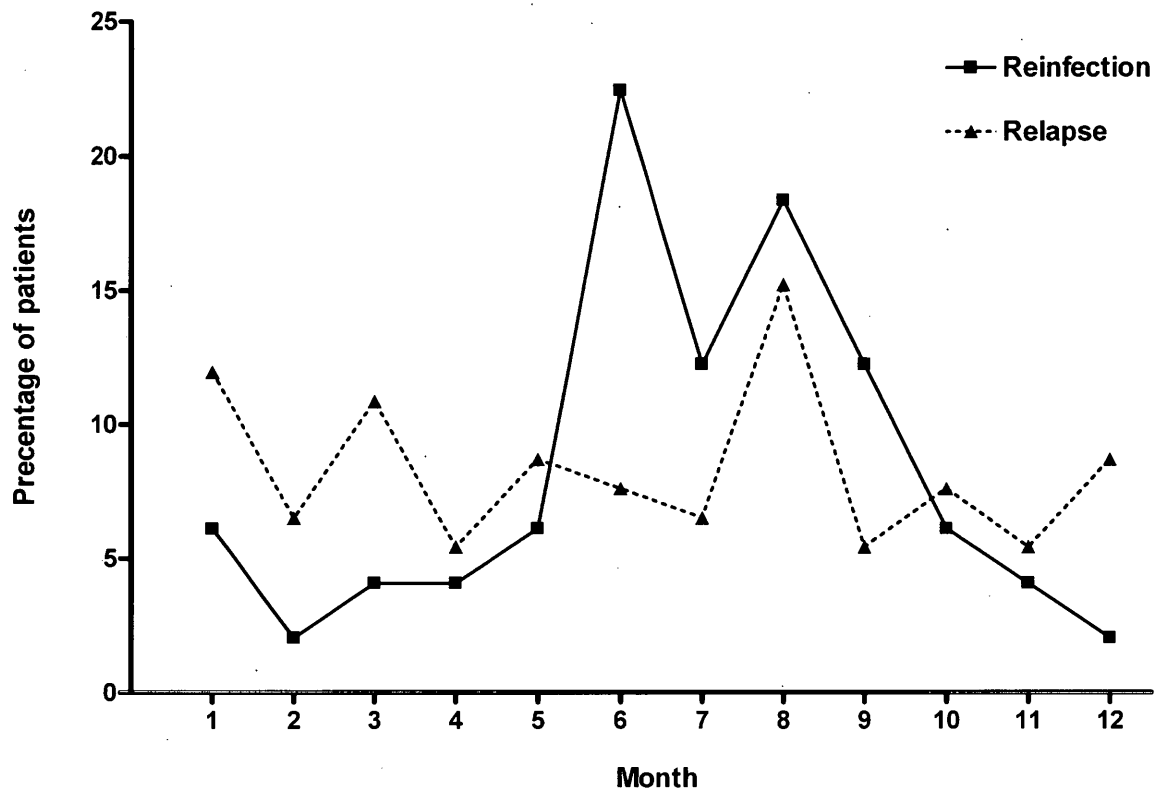
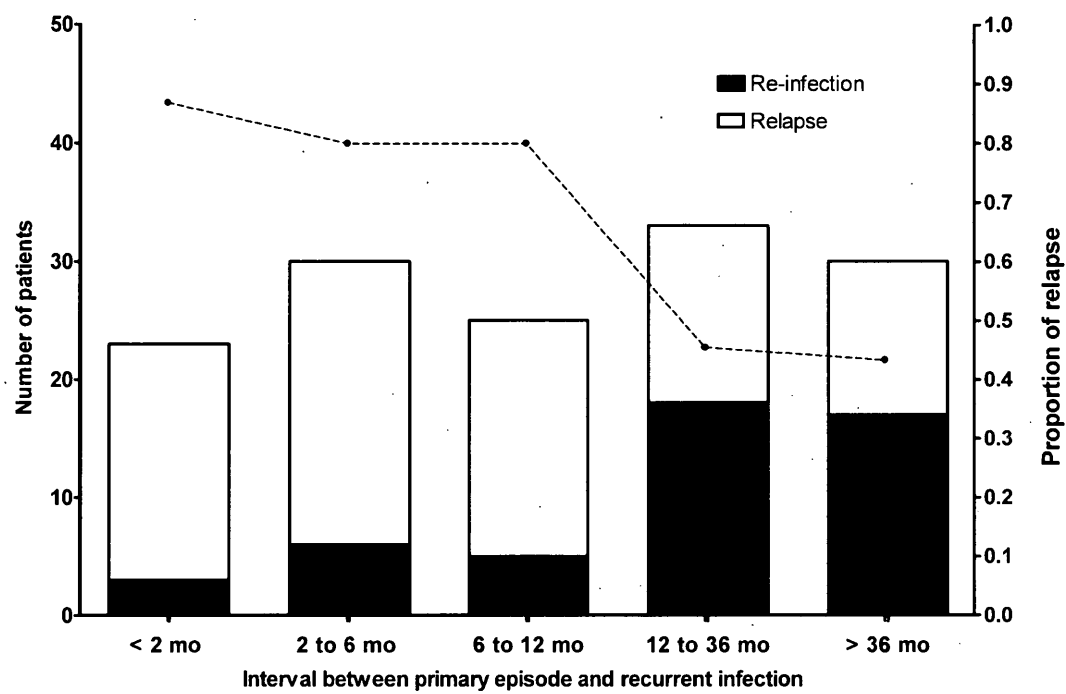


Figure 7-2. Interval between primary episode and recurrent infection for patients with relapse or reinfection.



7.3.5 Scoring system to determine cause of recurrent melioidosis.

A scoring system was generated based on a combination of predictors of re-infection or relapse in the multivariate model (figure 7-3). Factors associated with re-infection (presentation during the rainy season or with reduced renal function) were given a positive score. An arbitrary distinction was made between an estimated GFR on admission of < 30, 30 to < 60, 60 to < 90, and ≥ 90 mL/min per 1.73 m^2 . Factors associated with relapse were given a negative score. The interval between the primary infection and recurrence was considered as being within one year, or greater than one year from the start of oral antimicrobial therapy. Oral antimicrobial treatment of the primary infection was grouped into periods of < 8 weeks, 8 to < 16 weeks, 16 to 20 weeks and > 20 weeks. A non-linear association was found between the duration of oral treatment received and predictive value of relapse. A score was reached based on the accumulation of points from the four variables. The area under the ROC curve for the re-infection score was 0.84 (95%CI, 0.77-0.91).

The predictive ability of the risk index model for relapse and re-infection is depicted in figure 7-4. A score of less than -5 correctly identified relapse in 75 of 89 patients (84%) in this group, whereas a score of more than 0 correctly identified re-infection in 29 of 40 patients (73%). A score ranging from -5 to 0 was classified as indeterminate; a total of 12 patients (9%) fell into this group.

Figure 7-3. Predictors of re-infection and relapse for patients with recurrent melioidosis.

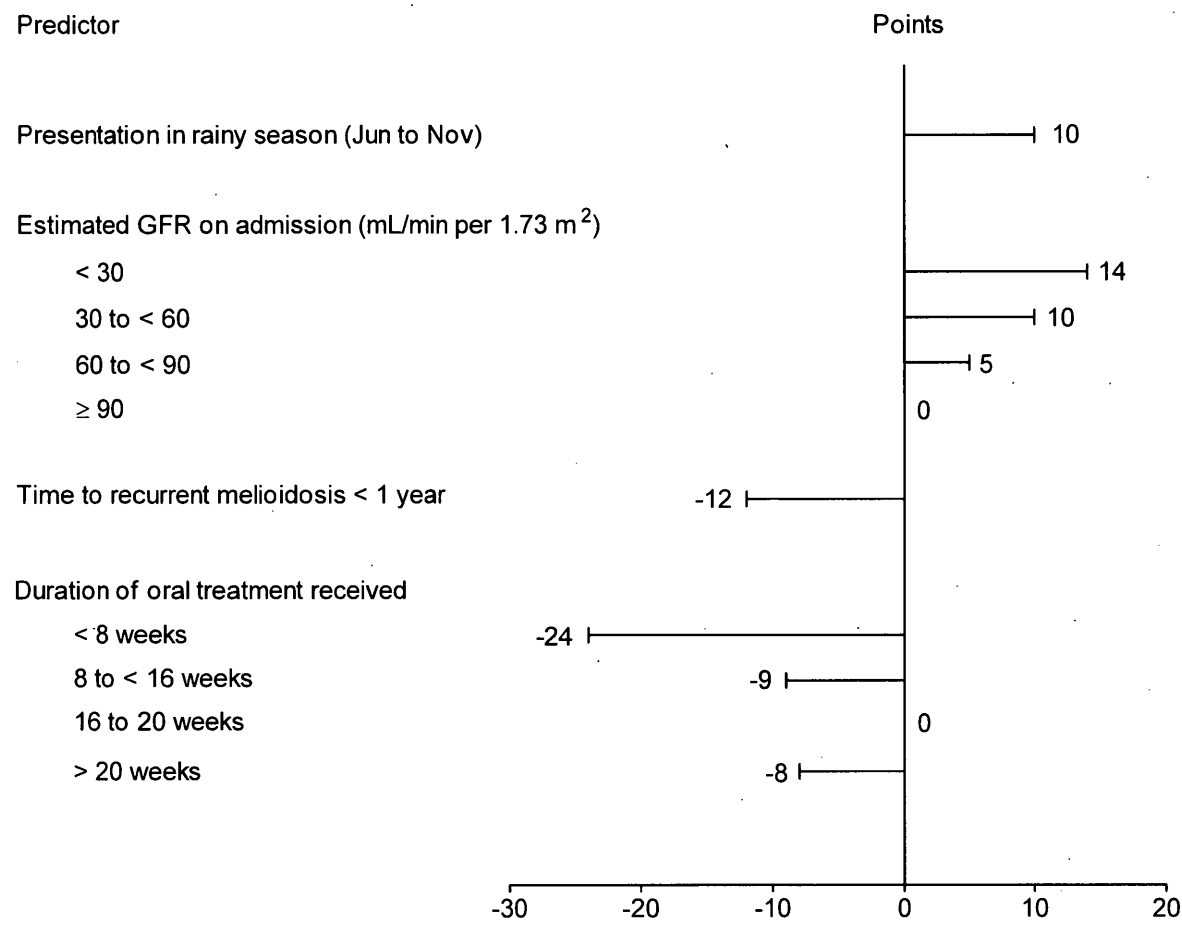
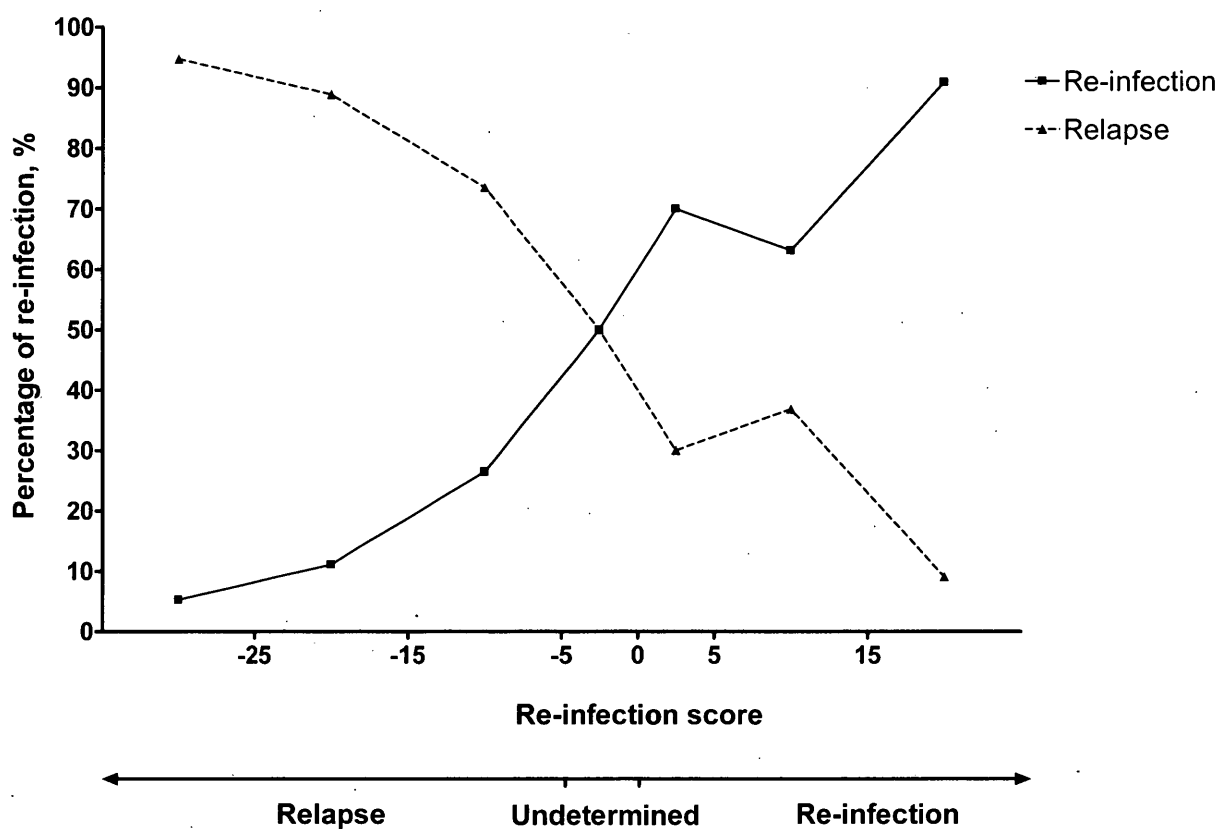


Figure 7-4. Predictive ability of the risk index model for relapse and re-infection
within range of point < -25, -25 to < -15, -15 to < -5, -5 to 0, >0 to 5, >5 to 15, and >
15, respectively.



7.4 Discussion

Determining the cause of recurrence in a range of infectious diseases is very important as relapse and re-infection have different implications for disease control and clinical management. Relapse reflects treatment failure, in which antimicrobial regimen, elimination of a persistent focus and drug adherence are the main concerns. Re-infection on the other hand involves exogenous infection with a new strain, and has implications for disease prevention and health education strategies. In clinical practice, if all recurrent infections are generally assumed to be relapses, inferior secondary treatment may be used based on the assumption that the recurrence is a failure of previously prescribed first-line treatment. Use of second-line drugs is associated with an increased rate of relapse (chapter 6), and exposes patients with re-infection to a higher risk of relapse from this new episode than would otherwise be the case. In addition, the prevention of re-infection remains ignored.

For many infectious diseases, the clinical differentiation of relapse from re-infection is difficult or impossible, and genotyping has been generally been used for this purpose. Examples include tuberculosis (45;141), malaria (121;168), *Staphylococcus aureus* bacteraemia (92), pneumococcal bacteraemia (91), infective endocarditis (52) and nosocomial infections (190;227). However, genotyping techniques are generally not widely available for tropical infections in endemic areas. In addition, isolates are rarely stored outside of the research setting, making it impossible to compare isolates associated with the primary and recurrent disease.

Clinical differences between re-infection and relapse have been proposed for Lyme disease, although a scoring system was not developed (162). Scoring systems have been described for the prediction of outcome from melioidosis (37), and to predict a number of other events including atrial fibrillation after cardiac surgery (155). This scoring system is the first clinically-based scoring system to differentiate between

relapse and re-infection in any infectious disease, and may act as a paradigm for other infections. It is fast and simple to use, necessitating data on only four easy to assess factors. This scoring index can be used where bacterial genotyping is unavailable, which covers the majority of melioidosis-endemic regions. The factors associated with recurrent melioidosis are similar to those reported for recurrence of Lyme disease (relapse after previous inadequate treatment and within a short period, and re-infection during the 'high' season when ticks increase in numbers) (162) and may represent features that could be used for other infectious diseases.

Using genotyping to compare primary and recurrent isolates to distinguish between relapse or re-infection could be confounded by two major factors. First, 're-infection' could actually represent relapse in the event that primary infection was caused by simultaneous infection with more than one bacterial strain, and different strains were picked by chance for genotyping (176). However, infection with more than one strain of *B. pseudomallei* occurs in less than 2% of case (chapter 4). Second, 'relapse' could actually represent re-infection in the event that re-infection was caused by a different strain that was nonetheless indistinguishable on genotyping from the first infecting strain. The probability of this occurring is unlikely, however, since the *B. pseudomallei* population in the environment is extremely diverse (chapter 5).

The finding of a non-linear association between duration of oral treatment received for the primary episode and predictive value of relapse is consistent with a previous analysis; patients treated for more than 20 weeks may have more complicated or severe disease associated with a higher risk of treatment failure and relapse (chapter 6). Bacteraemia and multifocal infection during the primary episode have been identified as risk factors for relapse compared to patients who did not have relapse (chapter 6); however, these two variables were not significantly different between the relapse and re-infection groups. This scoring system was developed in Thailand, and

validation in other settings such as northern Australia is required to determine its applicability elsewhere.

7.5 Chapter summary

This study highlights clinical factors associated with presentation of relapse and re-infection, and describes the development of a simple scoring system to define the probable cause of recurrent melioidosis. Duration of oral antimicrobial treatment received for the primary episode, time between the primary episode and recurrence, presentation of recurrence during the dry or rainy season and GFR at recurrence were independent predictors of relapse or re-infection. Factors associated with re-infection were given a positive score and factors associated with relapse a negative score. An overall score of less than -5 correctly identified relapse in 75 of 89 patients (84%), whereas a score >0 correctly identified re-infection in 29 of 40 patients (73%). The scoring index had good discriminative power, with an area under the receiver operating characteristic curve of 0.84 (95%CI, 0.77-0.91). This simple scoring index can be used to predict the most probable cause of recurrent melioidosis, providing timely and important bedside information. This could prove invaluable in settings where genotyping is not available and bacterial isolates are rarely stored. The findings have the potential to improve clinical care at zero cost, and could act as a paradigm for other recurrent infections.

Chapter 8. Concluding comments.

1. Genotyping was used to compare isolates pairs cultured during primary and recurrent melioidosis in 115 patients to determine the proportion of patients who have a subsequent episode of infection due to failure to eradicate the organism (relapse) versus those who become re-infected with a new strain. Three quarters of cases were shown to be due to relapse, and one quarter was related to re-infection. The incidence of re-infection in the study population after survival of a first episode of melioidosis was significantly higher than previously published figures for the general population in this area, a finding that has important implications for prevention. This increase in risk may be due to host genetic or acquired factors. On-going studies in our laboratory are evaluating host genetic susceptibility for melioidosis, in which genetic polymorphisms relating to innate and acquired immunity are being defined in melioidosis cases and controls. The events leading up to relapse may also relate to bacterial factors. Whole genome sequencing of two pairs of primary and relapse strains is being conducted by TIGR to determine the nature of genetic changes that accrue *in vivo* over time. A larger number of strain pairs could also be examined using variable number tandem repeats (VNTR) to determine the extent to which microevolutionary changes occur in the human host. An important, as yet unanswered question is where and how *B. pseudomallei* persists in the human body. *B. pseudomallei* are taken up by phagocytic cells, and it is possible that this results in bacteria becoming shielded from the immune response and the effect of antibiotics. Multinucleate giant cell formation has been noted to occur during human disease, and it is possible that bacteria become adapted to persist in this potentially complex cellular milieu. Unpublished data from our laboratory indicates that relapse often affects the same body sites as those implicated in the primary episode. This could be interpreted to mean that the organism persists at the

infected site(s) and leads to relapse affecting the same organ. However, involvement of the same organ has also been observed for patients with re-infection. This suggests that host susceptibility may play a major role in the body sites involved in both relapse and re-infection, either because seeding of bacteria is more likely in previously damaged tissue, or because host genetic traits increase the probability for a specific pattern of organ involvement and disease manifestations. Mouse models and host genetic studies in patients with recurrent infection may ultimately provide answers to these questions.

2. Genotyping of isolates from primary and recurrent melioidosis could be prone to error if mixed infection was common during primary infection, and different strains were picked by chance during the first and subsequent episode for genotyping. In this case, relapse would be mistakenly classified as re-infection. A large prospective study of 133 patients with culture-proven melioidosis was performed to determine the rate of infection with more than one strain of *B. pseudomallei*. This proved to be rare, and so does not represent a significant source of error.

3. Assigning re-infection based on the finding that the isolate cultured during primary and recurrent melioidosis was the same genotype could be prone to error if the population genetic structure of *B. pseudomallei* in the environment, where infection is acquired, was clonally restricted. In this case, re-infection could be mistakenly misclassified as relapse. A study was conducted to define the genetic diversity of *B. pseudomallei* in soil in an area of disused land in NE Thailand. This demonstrated that environmental *B. pseudomallei* are highly genetically diverse, and it is highly improbable that a patient would be re-infected by chance with the same strain. An ongoing study is now evaluating the genetic diversity of *B. pseudomallei* in a nearby rice field, to reflect the type of setting where the majority of infections are probably

acquired. Preliminary data indicate that marked genetic diversity is also observed, despite the effects of ploughing, fertilizers and pesticides.

4. Understanding the risk factors for relapse and re-infection could result in strategies to reduce the risk of these events. Multivariate analyses identified choice and duration of oral antimicrobial therapy as the most important determinants of relapse, followed by positive blood culture and multifocal distribution. This has important implications for patient care. Compliance with oral medication taken for the treatment of melioidosis is likely to be crucial, in addition to appropriate prescribing practices by medical staff. None of the factors considered were found to be risk factors for re-infection, but targeting behavioural factors that lead to repeated exposure to the organism is a realistic option for reducing risk in this group. General guidelines to reduce exposure have been recommended to farmers, who are the highest at-risk group. However, rice farming involves wading in mud together with exposure of the arms, and wearing full-length boots and gloves may prove uncomfortable during the course of a working day in the tropics. Alternative strategies are an increase in the use of mechanical implements to reduce exposure time, and eradication of *B. pseudomallei* from the soil. The former requires analysis of cost-effectiveness and government support as it is expensive. For the latter, studies of the efficacy of biocontrol are being conducted. The ultimate in prevention would be the development and implementation of a vaccine programme. However, this may prove to be more complex than for other organisms. Most healthy individuals living in melioidosis-endemic areas in Thailand develop antibodies to *B. pseudomallei* but these are not protective in later life. Since *B. pseudomallei* is an intracellular pathogen the development of cell-mediated immunity (CMI) is likely to be important, and it is not clear whether healthy exposed people also develop CMI that goes on to fail in later life. A vaccine would need to protect the most

vulnerable individuals with co-morbidities such as diabetes and chronic kidney disease, and this may represent a significant challenge. Testing such a vaccine would also be a challenge, since the identification of surrogate markers of protection such as an antibody response or CMI may not be sufficient to demonstrate efficacy, and large studies would be required that were sufficiently powered to show a reduction in disease incidence. Further important insights may be gained by studying the immune responses of patients who do and do not relapse or become reinfected following a primary episode of melioidosis.

5. The majority of hospitals in melioidosis-endemic regions do not have access to genotyping facilities. A simple scoring system was devised that utilises readily available information to determine the most probable cause for recurrence. This approach may be applicable to other infectious diseases in which recurrent infection occurs following a primary episode, including tuberculosis (45;141), malaria (121;168), *Staphylococcus aureus* bacteraemia (92), pneumococcal bacteraemia (91), infective endocarditis (52) and nosocomial infections (190;227). This is complicated by the fact that simultaneous infection with more than one genotype is not uncommon in malaria and tuberculosis. In addition, a genotyping scheme for malaria has yet to be evaluated and standardized. Specific risk factors for relapse and re-infection in these infectious diseases have not been fully evaluated although this could prove useful for patient management. As genotyping is not available in resource-limited settings and isolates are rarely stored outside of the research setting, a simple scoring system to determine the cause of recurrent infection in other infectious diseases could have widespread applicability.

Chapter 9. References.

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Appendix. Solutions and buffers for PFGE.

1 M Tris (pH 8.0)

121.1 g of Tris base

Dilute to 1,000 ml with distilled water and adjust pH with HCl to 8.0

0.5 M EDTA (pH 8.0)

186.1 g of EDTA

Dilute to 1,000 ml with distilled water and adjust pH with NaOH to 8.0

SE buffer (75 mM NaCl and 25 mM EDTA, pH 7.5)

4.38 g of NaCl

50 ml of 0.5 M EDTA (pH 8.0)

Dilute to 1,000 ml with distilled water and adjust pH to 7.5

Note: The SE buffer is used to make the plug agarose.

Lysis buffer (0.1% sodium dodecyl sulfate and 25 mM EDTA, pH 8.0)

1 g of sodium dodecyl sulfate

50 ml of 0.5 M EDTA (pH 8.0)

Dilute to 1,000 ml with distilled water and adjust pH to 8.0

TE buffer (10 mM Tris and 10 mM EDTA)

10 ml of 1 M Tris (pH 8.0)

20 ml of 0.5 M EDTA (pH 8.0)

Dilute to 1,000 ml with distilled water.

Note: The TE Buffer is used to wash lysed PFGE plugs.

5x TBE buffer (450 mM Tris, 450 mM boric acid, and 10 mM EDTA)

54 g of Tris base

27.5 g of boric acid

20 ml of 0.5 M EDTA (pH 8.0)

Dilute to 1,000 ml with distilled water

0.5x TBE buffer (45 mM Tris, 45 mM boric acid, and 1 mM EDTA)

100 ml of 5x TBE buffer

900 ml of distilled water

Note: The 0.5x TBE buffer is used for gel electrophoresis running buffer.